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Title: **Characterisation of phosphotransferase systems of *Clostridium beijerinckii* belonging to the mannose/fructose/sorbose family**

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List of Abbreviations

A	adenine
ABE	acetone-butanol-ethanol
amp	ampicillin
ATP	adenosine 5’ -triphosphate
BLAST	Basic Local Alignment Search Tool
bp	base pair
C	cytosine
^{0}C	degree Celsius
cm	centimetre
dH_2O	distilled water
G	guanine
M	Molar
kan	kanamycin
mg	milligram
ml	millilitre
µl	microlitre
mM	millimolar
mm	millimetre
PTS	phosphotransferase system
rpm	revolutions per minute
T	thymine
TAE	Tris-acetate-EDT

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Abstract

After many years of decline, the production of acetone, butanol and ethanol via bacterial fermentation (ABE fermentation) is gathering renewed interest from those in the fuel industry. This is due to an increasing awareness of the damages fossil fuels have on our environment and also their limited supply. Species classified within the genus *Clostridium*, particularly *Clostridium acetobutylicum* and *Clostridium beijerinckii*, are long time favourites for use in ABE production. In order to re-establish the ABE fermentation process a better understanding of the physiology and metabolism of these strains is required.

The PEP-dependent phosphotransferase system (PTS), which catalyses both uptake and phosphorylation of its substrates, is a major mechanism of sugar uptake in anaerobic bacteria. The genome of *Clostridium beijerinckii* 8052 encodes a total of 43 complete phosphotransferase systems, nine of which are categorised as belonging to the mannose/fructose/sorbose superfamily. Genes encoding four of these nine systems were amplified by PCR and transformed into *Escherichia coli* ZSC113, a strain incapable of phosphorylating mannose and glucose, in order to determine the transport function of each system via complementation of the fermentation phenotype on indicator agar. The PTSs encoded by *cbei*0712-0713, *cbei*3871-3874 and *cbei*0957-0958 all gave negative fermentation phenotypes for both mannose and glucose. PTS *cbei*0965-0966, however, gave a positive fermentation phenotype for mannose, but not for glucose.

As the strain *E. coli* ZSC113 was isolated 40 years ago following chemical mutagenesis, the exact positions of mutations have been unknown. Therefore the *manXYZ* genes encoding the PTS, responsible for transporting mannose, glucose and other sugars, were amplified by PCR and sequenced to search for mutations. It was found that one mutation lay in *manX*, three in *manY* and one in *manZ*.

1. Introduction:

Acetone-butanol-ethanol (ABE) fermentation is a process whereby solventogenic bacteria produce acetone, n-butanol and ethanol from a natural carbon source. These bacteria were once exploited to produce large volumes of solvents in the early 20th century, but as recovering petroleum based products became more cost effective, the ABE industry declined (Bahl and Durre, 2001, Chapter 1.2). Now that it is recognized that the use of fossil fuels has harmful effects on the environment and that they are in limited supply, interest in producing fuel via ABE fermentation has been revived. In Europe, a target has been set to raise the percentage of biofuels used in all automotive transport to 10% by 2020 and is projected to reach 25% in Germany by that time (Tilche and Galatola, 2007; Durre, 2007).

The most commonly used bacterial species for ABE fermentation are *Clostridium acetobutylicum* and *Clostridium beijerinckii*, however other species are also known to use this fermentation pathway. There are several issues that make large scale ABE production too costly to compete with fossil fuels in the market. One of these issues is that the toxicity of butanol to clostridia which leads to sporulation when the butanol concentration rises, limiting the production of each batch. A larger problem is the high cost of substrate and also the diversion of these substrates into products other than butanol. With a greater understanding of the mechanics behind carbon absorption, this will hopefully lead to an increased uptake of carbon per batch and consequently a higher production rate of ABE.

Ethanol, currently the biofuel manufactured in the largest quantities, is produced by yeast which cannot metabolise many of the sugars present in alternative biomass sources, such as food waste (Qureshi and Ezeji, 2008). In contrast to butanol, ethanol can be produced in high concentrations, making it more cost effective to manufacture.With a greater understanding of all parts of the fermentation process, it is hoped that these issues can be overcome and that butanol and ethanol can be used widely as a source of fuel. This study will focus on the carbon absorption stage in *C. beijerinckii*, specifically uptake of mannose.

1.1 A History of *ABE Fermentation*:

ABE fermentation was first discovered by Louis Pasteur in 1861 (Pasteur, 1861a and b). He named his fermenting bacterium "*Vibrion butyrique"*, thought today to be a clostridial species due to its butanol producing abilities and its inability to tolerate oxygen (Bahl and Durre, 2001:Chapter 1.2). Later in the 19th century, several researchers investigated the production of butanol by anaerobic bacteria (Killeffer, 1927;McCutchan and Hickey, 1954; Prescott and Dunn, 1959), whereas acetone production was not reported until 1905 (Schardinger, 1905).

However bacteria were not used for the production of solvents on an industrial scale until between 1912 and 1914 Chaim Weizmann discovered that a strain he called "BY" (now known as *Clostridium acetobutylicum)* which was capable of producing large yields of butanol and acetone, using many kinds of starches under the required conditions (Gabriel, 1928, 1930). Weizmann was a chemist working on developing the synthesis of rubber and had believed that butanol production played an essential role in its synthesis. He worked with Fernbach and Scheon of the Institute Pasteur to investigate the production of butanol using microbial fermentation. While Fernbach had isolated a strain in 1911 that was able to ferment potatoes into butanol, Weixmann's BY could ferment a variety of starchy substances into butanol and produced better yields (Gabriel, 1928, 1930. Hastings, 1978). He then developed a method to use ABE fermentation on a large scale to produce quantities of acetone and transferred the rights to his method to the Commercial Solvents Corporation in 1914 (Hughes, 2009). This act excluded his competitors from using the applied AB process until the end of 1935.

Acetone was used in the production of cordite explosive propellants in the First World War which played a crucial role to the Allied war effort. Butanol was seen as an unwanted by-product and was put into storage throughout the war. Acetone production declined at the end of the war as the demand for it decreased and attempts were made to salvage the butanol that had been stored when a new use was found for it. The demand for butanol soon increased as the automobile industry in the USA began using it as a starting material to produce butyl acetate, a material used to make quick-drying paint for cars (Rose, 1961). Research at that time began focusing on developing a new strain which would be able to utilise maize mash as a carbon source for ABE fermentation. While many new strains

were found to produce ABE, the results were not to the same standard set by Weizmann's strain (Hastings, 1971).

In the 1930s, molasses was commonly used instead of maize mash as a low-cost alternative raw material. This subsequently played a role in the development of ABE fermentation technology. Molasses were first used as a commercial substrate in 1932 by the Commercial Solvents Corporation (CSC) (Jones and Keis, 1995).
Once Weizmann's patent expired in 1936, several new ABE fermentations were established in the USA and Puerto Rico, competing with the CSC. Around this time, ABE processes were also developed in Japan, Australia, India and South Africa (Ross, 1961; Spivey, 1978).

The CSC developed a fermentation plant in Bromborough, England, shortly before the end of the patent. From the beginning of 1936 onwards ABE production from the fermentation of molasses rapidly progressed. Various new strains which were capable of carrying out the fermentation process were isolated and owned by various companies (Beesch, 1952;. McCutchan and Hickey, 1954). Companies owned numerous strains with different properties with at least 18 patents being issued covering different strains between 1935 and 1941 (Beesch, 1952). McCutchan and Hickey,1954). In the United Kingdom once World War II began, the production of ethanol from molasses was virtually stopped, but since acetone was in high demand for the manufacture of munitions, ABE fermentation was given top priority (Hastings, 1978). The demand for acetone increased as the war progressed and the fermentation plant in Bromborough was expanded and after the war, resumed normal commercial operations. However, the plant soon found itself struggling to compete with chemically produced solvents. Due to a high increase in the price of molasses, the plant began to use cheaper beet-based molasses produced within the UK (Hastings, 1971). Despite this change, the competition lead to the eventual closing of the plant in 1957 (Gibbs, 1983).

In the United States of America, one tenth of total acetone and two thirds of the total butanol produced were still being produced by fermentation at the end of the war in 1945. The percentages of these solvents being manufactured by fermentation greatly reduced over the 1950s and had virtually ceased by the 1960s in both the USA and the UK (Rose, 1961). This was largely due to two reasons; the rapid growth of the petrochemical industry

during the 1950s proved to be fierce competition to the fermentation industry. Secondly, molasses were becoming a popular ingredient in cattle feed and large amounts were being used for this purpose, causing their price to escalate making them a far more expensive substrate (Hastings, 1978).

In the USSR, acetone and butanol were produced on a fairly large scale where wheat, rye and maize were used as raw materials for ABE fermentation (Hastings, 1971,1978; Nackhmanovich and Shcheblykina, 1959). This changed in the late 1950s when attempts were made to use other substrates such as mixtures of molasses and corn cob hydrolysates following a decision made by the 20th Congress of the Communist Party (Nackhmanovich and Shcheblykina, 1959). Several reports were published in the 1950s and 1960s on ABE production by continuous fermentation which lead to a factory scale process being put into effect in Dokshukino, 1960 (Yarovenko, 1964).
In Germiston, within the maize growing part of South Africa, a plant producing acetone and butanol using maize mash was established in 1937 (Robson and Jones, 1961 and Rose, 1961). Towards the end of the Second World War the plant converted to using molasses rather than maize mash and continued to produce solvents until it ceased operating in 1983.

ABE fermentation continued in China until the 1990s, when it finally gave in to its petrochemical competitors and ceased production. However, due to concerns about environmental pollution and the limited supply of fossil fuels, several ABE fermentation plants in China resumed production in 2006 (Ni and Sun, 2009). Shortly after its reintroduction, total ABE solvent production capacity from ten plants was 210,000 tonnes and was expected to rapidly increase to 1,000,000 tonnes in 2008.

1.2 The Carbon Cycle:

Carbon is a ubiquitous element on the planet Earth and plays a crucial role for all forms of life upon it. While there is approximately 10^{17} tons of carbon on the planet, a mere 0.05% is free to actively participate in the carbon cycle while the rest is embedded in the Earth's lithosphere (Sitch *et al.*, 2003).

The carbon cycle is the biogeochemical cycle whereby carbon is transferred between the biosphere, geosphere, pedosphere and hydrosphere. One major driving force behind the cycle is living organisms as they absorb and release carbon in various ways. This includes the amount of carbon transferred by human activity, such as the burning of fossil fuels and mining (Figure 1.1). Carbon is also distributed by natural forces, such as the force of the wind and by ocean movements, but its chemical structure remains largely the same compared to distribution via live organisms.

Carbon is absorbed into plants from the atmosphere and from decomposing organisms in soil. This carbon is arranged into carbohydrates in order to store energy gained from photosynthesis. These plants can then be eaten by animal life and stored in their bodies as fat and as various forms of carbohydrate. When metabolised, carbon is then released into the atmosphere as CO_2. Carbon is also present in faecal animal waste which is returned to the soil and degraded by bacteria of the rhizosphere to participate in the cycle again. Carbon is also released back into the soil when the animal dies. Carbon from the dead remains of plants and animals can either be metabolised by bacteria and/or fungi and returned to the cycle or it can be stored in the soil in the form of fossil fuel (peat, coal, oil).

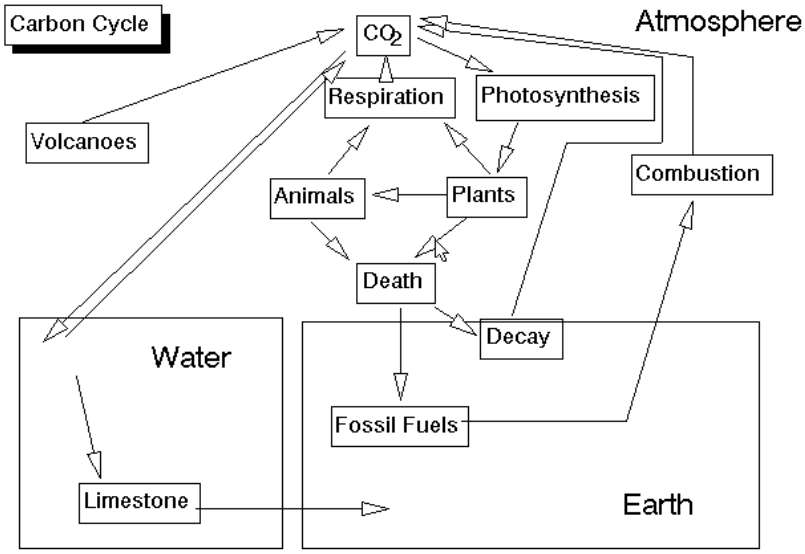


Figure 1.1: An illustration of the carbon cycle. This diagram is courtesy of David McShaffrey of Marietta College.

1.3 Energy and Fuel:

Sources of energy can be divided into three categories; fossil, renewable and nuclear. Over the last 10,000 years humans have been using various kinds of biomass as fuel to sustain themselves in order to travel, cook, eat and warm their homes. Early humans used comparatively simple technology which did not reach high energetic efficiencies and thus did not require copious amounts of fuel. They mainly used renewable sources such as wood, hydropower (watermills) and wind power (windmills), however within the last 200 years attention has shifted to the use of fossil fuels through the use of mining. Industrialisation meant that our energy requirements could no longer be fulfilled solely through the use of simple renewable sources. Sources of fossil fuel energy include coal, petroleum, oil shale, natural gas, bitumen and tar sands.

Fossil fuels contain a higher density of energy compared to their more primitive renewable counterparts and could sustain newly developed technology such as the steam-driven machines used in factories and steam powered trains. It was generally assumed that fossil fuels would forever be abundant so it appeared that there was no need to curb their use. As of 2010, petroleum is the largest single source of energy being consumed by Earth's

population at an average of 4.8 barrels[1] per person per year (Gupta and Demirbas, 2010), exceeding the use of coal, natural gas, nuclear energy and hydroelectric power. However, it has recently been realized that fossil fuels cannot be replaced as rapidly as they are being consumed and attention has been drawn back to renewable sources and ways in which to make them as economical as the use of fossil fuels currently is. While efforts are being made to reintroduce biofuels and other renewable sources, over 88% of global energy used in 2009 was derived from fossil fuels (Gupta and Demirbas, 2010).

1.4 Issues with Fossil Fuels:

In 2008, fossil based fuels made up just over three quarters (76%) of total energy consumption around the world (Stöcker, 2008). According to Stöcker, the three main sources of energy used were petroleum (33%), coal (24%) and natural gas (19%). Alternatives to fossil-based fuels that were used to a significant extent included biomass (13%), hydropower (6%) and nuclear power (5%). As supplies of fossil-based fuels decrease and as the effects they are believed to be having on the environment is raising increasing concerns, it is essential to increase the use of renewable energy sources over the next few years.

The mining of coal, both by opencast and underground methods, has a negative impact on the surrounding environment (Dhar, 1993). Underground mining is a major source of pollution to local rivers and streams. This occurs when water floods into the mines and is then channeled into surrounding rivers and streams which become chemically polluted (Tiwary and Dhar, 1994). Preparation plants and coal beneficiation, the process whereby the coal product is made as homogenous as possible, also generate water effluent which affects the aquatic ecosystem when it reaches these rivers, resulting in a reduction of local biodiversity (Tiwary and Dhar, 1994). Other ways in which coal mining causes water pollution are by drainage from the mining sites, including acid mine drainage, and by mine water and sediment runoff (Tiwary, 2000).

[1] While a barrel unit has not been universally standardised, it is defined in the US and Canada as being 42 US gallons (159 litres) in volume.

The international trade of crude oil is largely driven by politics which can make its prices and distribution highly unstable. A climactic series of events that occurred over the 1970s led to a dramatic increase in oil prices over the course of the decade. The price of a barrel of oil shot from just under $20 to close to $50 by 1975 and then to over $100 by the end of the decade at today's equivalent amount of USD (EIA, 2008).

Oil prices are highly susceptible to the political environment of their origins. A large amount of oil is produced in the Middle East where a series of revolutions and other conflicts have disrupted its trade from key global producers (Hamilton, 2009). While the Yom Kippur War in 1973 did not in itself prevent a significant number of oil shipments, it was announced by the Organization of Arab Petroleum Exporting Countries (OAPEC) that it would cut production by 5% until Israeli forces "are completely evacuated from all the Arab territories occupied in the June 1967 war, and the legitimate rights of the Palestinian people are restored." (Hamilton, 2009). As biofuels can be readily produced in the country that uses them, they are not susceptible to the same political and economic instability that crude oil and other fossil fuels are.

1.5 Biofuels:

Biofuels are an alternative to fossil fuels that are produced by living organisms. They are of interest as plant biomass is both a renewable and abundant source which can be produced in a relatively short amount of time compared to their fossil fuel counterparts which can take thousands of years to form. Until 2006, the only biofuel that was being produced on an industrial scale was bioethanol (Antoni *et al*, 2007), which is currently the most used for transportation worldwide (Balat and Balat, 2009). Other biofuels of interest include biomethane, biobutanol and biodiesel.

1.5.1 Biomethane:

While biomethane is produced on a large scale, it is not yet used as a fuel for transportation (Antoni *et al*, 2007). The concept of utilising biomethane to replace fossil fuels is an attractive one as it is already produced from animal waste (Tilche and Galatola, 2008), and is produced naturally in the intestines of mammals by methanobacteria. Methane acts as a greenhouse gas when left untreated and contributes significantly to

greenhouse gas emissions, leading to global warming and climate change. An abnormally high level of methane is produced from bovine waste due to the large population of cows kept on farms in the Western Hemisphere. By using these emissions as a fuel source, the biomethane could potentially reduce greenhouse gas emissions, rather than contribute to them (Tilche and Galatola, 2007). While methane is an excellent source of fuel for cooking, and heating water within the home in its gaseous form, it is not so suitable for use in combustion-powered vehicles. In order to use methane for such applications, it must first be transformed into methanol. This can be achieved by using partial oxidation to separate the methane into hydrogen and carbon monoxide at high temperatures before it is catalytically converted to methanol over a platinum or copper surface (Parravano *et al*, 1942). It is not the most efficient of methods since a large amount of the gas naturally oxidises into carbon dioxide and water, leaving around only 5-10% to be converted to methanol (Fajula *et al*, 1982). While methanol tends not to replace petroleum in motorised vehicles, it can be blended with it to produce cleaner fuel. Flexible-fuel vehicles or "flexi" vehicles can be used for this purpose as their internal combustion engines are designed to run on more than one fuel. Usually petroleum is blended with other fuels, methanol and ethanol being the most popular. Both fuels are stored in the same tank.

1.5.2 Ethanol:

Ethanol and biodiesel are commonly used in motorised vehicles to replace petroleum as a fuel source; however there are several disadvantages to using them. For the sole use of ethanol, a vehicle requires a differently structured engine than those designed to use petroleum. This means that vehicles must either be specially designed for the use of ethanol when purchased or an individual would have to pay a large fee to have their engine replaced or altered. While not frequently used as the sole source of fuel in vehicles, it is often used as an additive to petroleum in flexi vehicles. It is particularly popular in the USA where corn based ethanol can be produced locally and gives a political advantage. However, while more readily available than methanol blends, petroleum-ethanol blends are relatively scarce in comparison to the availability of pure petroleum and diesel products. Like methanol, ethanol burns at lower temperatures and is less volatile than petroleum, making it more difficult to start an engine in cold weather. Ethanol is a corrosive solution which can lead to the erosion of engine interiors if used in its pure form, unless the engine is designed to run on 100% ethanol. However, it can be successfully blended with other fuels such as petroleum and diesel, reducing the level of pollution

when used in this way. Less SO_2 and CO are emitted than when petroleum and diesel are used on their own in motor engines. A blend with ethanol also improves the efficiency of an engine cycle when compared to pure petroleum (Dodić *et al*., 2009). Ethanol is largely produced on an industrial scale by yeasts rather than clostridia. While these yeasts can efficiently produce ethanol, they cannot catabolise many of the sugars and starches found in plant waste materials and require grains, fruits and sugarcane for fermentation. This is less desirable as all these substrates can be used for human consumption and require stretches of farmland to grow. If plant wastes could be used, no choice would have to be made as to whether space should be used to grow plants for food or for fuel (Jacobs, 2007).

1.5.3 Diesel:

The use of biodiesel as a fuel is more environmentally friendly than its fossil fuel counterpart in several ways, but is not entirely carbon neutral. It is considered to be safer and less toxic than petroleum diesel during burning (Demirbas, 2009). It can only be used in engines built for diesel use, but can be blended with other fuels. There are however, some disadvantages to using biodiesel compared to petroleum based diesel. Biodiesel emits a high level of nitrogen oxide, has a high viscosity and lower energy content than petroleum diesel. It also gives a lower engine speed which can damage it with repeated use (Balat, 2011).

1.5.4 Butanol:

Butanol is a 4-carbon alcohol, commonly found in the form n-BuOH (normal butanol) and less frequently in the form of i-BuOH (isobutanol). Both forms can be used as an effective fuel (Ezeji et al, 2012).The use of butanol has several advantages compared to the use of ethanol as a fuel source. While the energy content of butanol is typically much lower than either diesel or biodiesel, it has a higher energy content per volume than ethanol and has a similar content to petroleum, the most popular fuel used in motorised vehicles (Table 1.1). Butanol is also less hydrophilic and less corrosive than ethanol.

While butanol makes a good source of fuel, there are several challenges in production that need to be overcome if it is to compete with other fuel types. As discussed previously, the most prominent of these issues currently include the high cost of substrate and the diversion of these substrates into less favourable products, such as acetone. Another

problem in butanol production is its toxicity to microbes. A concentration of 1% butanol can significantly inhibit the growth of the solventogenic bacteria producing it. Due to its toxicity to these microbes, the overall concentration of butanol rarely exceeds 1.5% during production, limiting the amount that can be produced per batch (Ezeji et al, 2012).

Table 1.1 Typical energy value of each fuel source.

Fuel Source	Energy Content (BTU per Gallon)
Petroleum	114,800
Diesel	140,000
Biodiesel; waste vegetable oil	120,000
Propane	92,500
Butane	130,000
Methanol	55,600
Ethanol	76,100
Butanol	110,000

Energy contents are displayed as British thermal units (BTU) per gallon (Fuels and Lubes International,2006, Ezeji et al, 2012 and Bartok 2004).

1.6 Solventogenic Clostridia:

Clostridia are gram positive, endospore forming bacteria that thrive in an anaerobic environment. They are described as being firmicutes along with *Listeria, Staphylococcus, Bacillus, Streptococcus* and some other genera (Bahl and Durre, 2001). While some species are responsible for serious human diseases, such as *Clostridium tetani*, causing tetanus, others can be exploited for human use. The solventogenic species of clostridia have been used in ABE fermentation for over a century in order to produce acetone and butanol in large quantities, whilst also producing ethanol as a minor product. They anaerobically catabolise both simple and complex carbohydrates which leads to the formation of these solvents as waste products. The principal species of clostridia known for their ability to form solvents are; *Clostridium acetobutylicum, Clostridium beijerinckii, Clostridium saccharoperbutylacetonicum* and *Clostridium saccharobutylicum (*Keis *et al*

1995). The genomes of all of these solventogenic clostridia have now been sequenced, although only two were available until recently (Papoutsakis, 2008; Mitchell, 2015). This has been key in helping us to better understand their abilities.

The species which was most popular for use in ABE fermentation was *C. acetobutylicum*, which could produce the largest quantities of butanol per batch.
Many different strains have been discovered and developed throughout the ABE fermentation process's relatively short history. The Commercial Solvents Company was responsible for the development of many early *C. acetobutylicum* strains while its patent excluding competitors from operating the fermentation, was active until the end of 1935 (Jones, 2001). Three particular strains were commonly used in Chinese factories before the petroleum and oil industries diminished their efforts. *C. acetobutylicum* AS 1.70, the wild type as well as *C. acetobutylicum* NA-Z and *C. acetobutylicum* EA 2018 which had various phage resistant properties and a particularly high percentage of butanol output respectively (Sun and Shi, 2006). Strain *C. acetobutylicum* EA 2018 has an average solvent output of 20.95% acetone, 71.45% butanol and 7.6% ethanol compared to the wild type's 30% acetone, 58% butanol and 12% ethanol (Sun and Shi, 2006).

While *C. acetobutylicum* has been the more popular species to use in ABE fermentation, it is not necessarily the best. For example, *C. beijerinckii* is less subject to degeneration of solvent formation, is more tolerant to fermentation inhibitors and has a wider substrate range than *C. acetobutylicum* (Mitchell, 2015).

1.7 *Clostridium beijerinckii*:

C. beijerinckii has the ability of utilising carbohydrates from several sources and can utilise a wide range of carbohydrates, especially when compared to *Clostridium acetobutylicum*. For these reasons it is of great interest to the ABE fermentation industry. As previously mentioned, one of the reasons for the high process cost of ABE fermentation was the cost of substrate due to fairly pure sources being used, such as corn and root vegetables, which can be used for human and animal consumption. Plant wastes which cannot be fed to livestock are a potentially suitable substrate as they are rich in carbohydrates and cheap to purchase. Plant waste has a much greater variety of starches and sugars than a pure source and so the microorganisms used to ferment it need to have

the ability to utilise a number of carbohydrates to make best use of the substrate available. The genome sequence of *C. beijerinckii* NCIMB 8052 was finally completed in 2007 and so it is now possible to characterise its genes based on their genetic similarity to those in other species which have already been fully identified. This project will focus on the strain *C. beijerinckii* NCIMB 8052. As in other anaerobes the phosphoenolpyruvate-dependent phosphotransferase system (PTS) plays a significant role in the utilisation of many sugars (Tangney *et al*, 1998) and so several of the PTSs of *C. beijerinckii* are the main focus of this study.

1.8 The Phosphotransferase System:

The phosphotransferase system (PTS) is a major carbohydrate transport system used by bacteria to accumulate extracellular sugars. Each PTS is responsible for the uptake of one or a group of related sugars. A bacterium lacking a particular PTS will be unable to uptake its associated sugar, unless it has an alternative method by which to do so.

The PTS is a multiprotein complex which uses phosphorylation to transport and initiate metabolism of sugars. The reaction results in a phosphate group being transferred from phosphoenolpyruvate (PEP) to a sugar, and involves several protein intermediates. These intermediates always include the general PTS proteins Enzyme I and Histidine-Containing Protein (HPr) and a membrane bound sugar specific complex Enzyme II. Enzyme II consists of several domains; A, B, C and occasionally D. Enzyme II domains may be associated with different proteins, or may be fused together in different combinations depending on the organism and system (Figure 1.2)

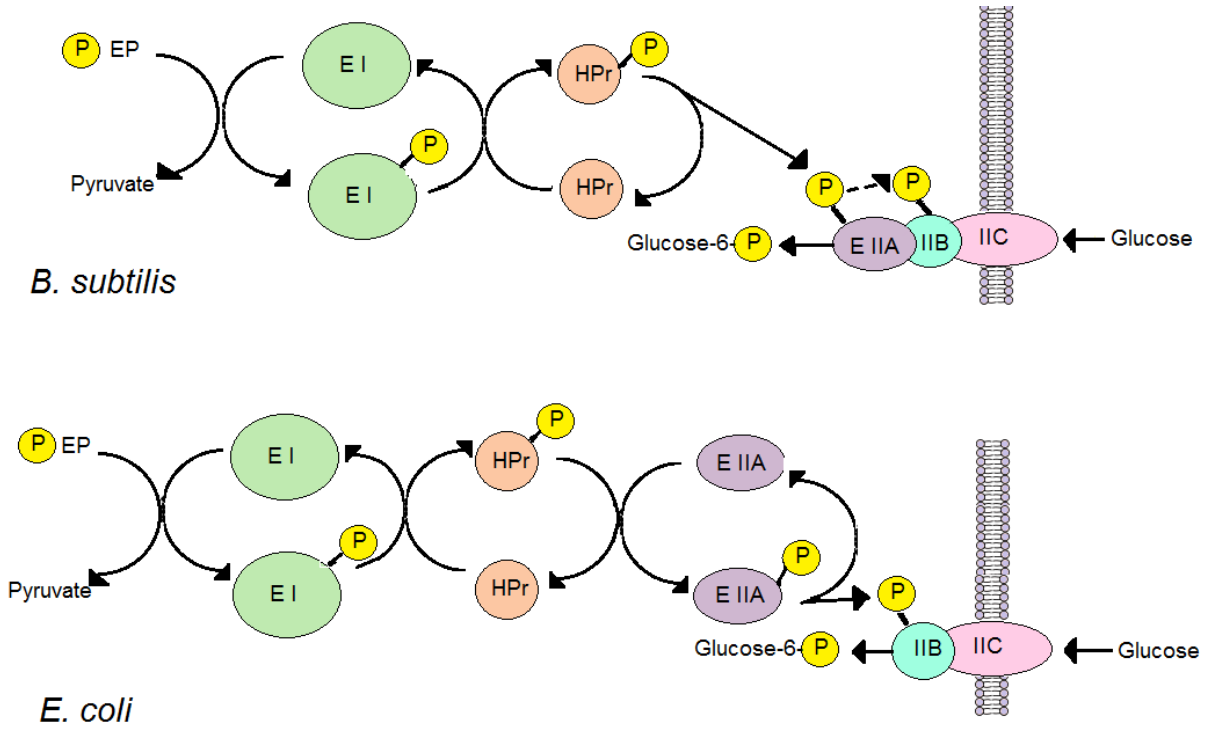


Figure 1.2 The glucose phosphotransferase system in Gram positive *Bacillus subtilis* and Gram negative *Escherichia coli*.

1.8.1: Enzyme I

Enzyme I is a general PTS protein, as it is nonsubstrate-specific and can be used by multiple systems responsible for the uptake of different carbohydrates (Lengeler *et al*, 1982). It is present in the cytoplasm, potentially allowing it to move freely within the cell. In the presence of Mg^{2+}, Enzyme I is autophosphorylated by PEP (Weigel *et al*, 1982). The phosphate group is then passed to HPr. Enzyme I is encoded by the ptsI gene. In many bacteria, although not the solventogenic clostridia, *ptsI* is part of the pts operon that also includes the *pts*H gene encoding HPr (Postma *et al*, 1993).

1.8.2: Phosphocarrier HPr

The phosphocarrier Histidine-Containing Protein (HPr) is a small protein which acts as the second protein in the phosphoryl transfer chain of the PTS. Its primary role is to transfer phosphate groups from Enzyme I to the Enzyme II complex (Singh *et al*., 2008). It was previously known as 'heat stable carrier protein due to its resilience to high temperatures,

but was renamed after the discovery that a phosphate is passed to other proteins via a histidine residue.

This protein consists of approximately 90 amino acids with a molecular mass of 9 to 10 kDa. In the phosphotransfer chain, HPr is phosphorylated on the residue His 15 and then passes the phosphoryl group to the Enzyme II complex (Postma et al., 1993). In Gram positive bacteria, it is also possible for HPr to be reversibly phosphorylated at a serine residue by a metabolite activated, ATP dependent protein kinase HPrK (Reizer et al, 1988). The *ptsH* gene encoding HPr has been identified in many bacteria and it appears that the expression of both genes *pts*I and *ptsH* are increased in the presence of different PTS sugars (Tanaka *et al.*, 2008; Tangney et al., 2003). It has been reported that the preference for glucose, which is considered the most effective sugar for the majority of these bacteria, can be due partly to an induction of the expression of these genes (De Reuse and Danchin, 1988; Stulke et al., 1997; Viana et al., 2000).

1.8.3: Enzyme II

Unlike Enzyme I, Enzyme II of the PTS is embedded into the cell membrane. Sugar specificity of the PTS resides in Enzyme II and bacteria usually possess many different Enzyme IIs, each specialised in the uptake of different carbohydrates. It consists of up to four separate proteins or domains, each with its own role in sugar transport. The Enzyme II complex always consists of two hydrophilic domains (IIA and IIB) and one or two hydrophobic domains which remain embedded into the cell membrane (IIC and IID). This gives them access to both the extra and intracellular environments and they form the channel by which the substrate of the PTS can enter the cell. PTSs are classified into separate families based on their genetic similarity and evolutionary origins gathered from phylogenetic data of EII (Saier *et al*, 2005). The Enzyme IIA and IIB domains act as phosphocarriers to the newly transported sugar molecules. The phosphate group transferred by Enzyme I and HPr is transferred to a histidine on Enzyme IIA and in turn passed to a cysteinyl or histidyl residue of the IIB domain. Once sugar has been transported though the membrane by IIC it is immediately phosphorylated by IIB, to retain the sugar in the cell for later use. An additional domain IID, is required for the uptake of some sugars, such as mannose.

1.9 Carbohydrate Uptake in Clostridia:

The PTS plays a vital role in the uptake of sugars in solventogenic clostridia, particularly in *C. acetobutylicum* and *C. beijerinckii* (Mitchell and Tangney, 2005; Mitchell, 2015). Other species of solventogenic clostridia have not been as closely studied in this respect.

The clostridial PTS functions in the same manner as the system in other bacterial species. As was shown *in vitro* by Mitchell *et al.* (1991) the soluble cell extract of the glucose PTS in *C. beijerinckii* NCMB 8052 successfully complemented the membranes of *C. pasteurianum, B. subtilis* and *E. coli* and vice-versa. This is particularly useful as it means that genes encoding several PTS of *C. beijerinckii* can be transferred into another species in order to attempt to determine their function via complementation of mutants. The *C. acetobutylicum* genome encodes 13 phosphotransferase systems (Barabote and Saier, 2005) while the *C. beijerinckii* genome encodes 43, nine of which belong to the mannose/fructose/sorbose family (Shi *et al,* 2010). All known PT-systems belonging to *C. beijerinckii* and their respective families can be seen in Figure 1.3. *C. beijerinckii* has PTSs belonging to all major families, only a few have been studied in any great detail. For example, the glucitol PTS combined with glucitol-6-phosphate dehydrogenase has been found to be induced by glucitol and repressed by glucose, and unusually it was found that its IIC domain was encoded by two proteins, one also carrying the IIB domain (Tangney et al, 1998). The mannose/fructose/sorbose family is of particular interest as the carbon sources transported by this family are found in many sources of biomass. A greater understanding of the systems belonging to this family will enable exploitation of *C. beijerinckii* for biomass utilisation in the ABE fermentation.

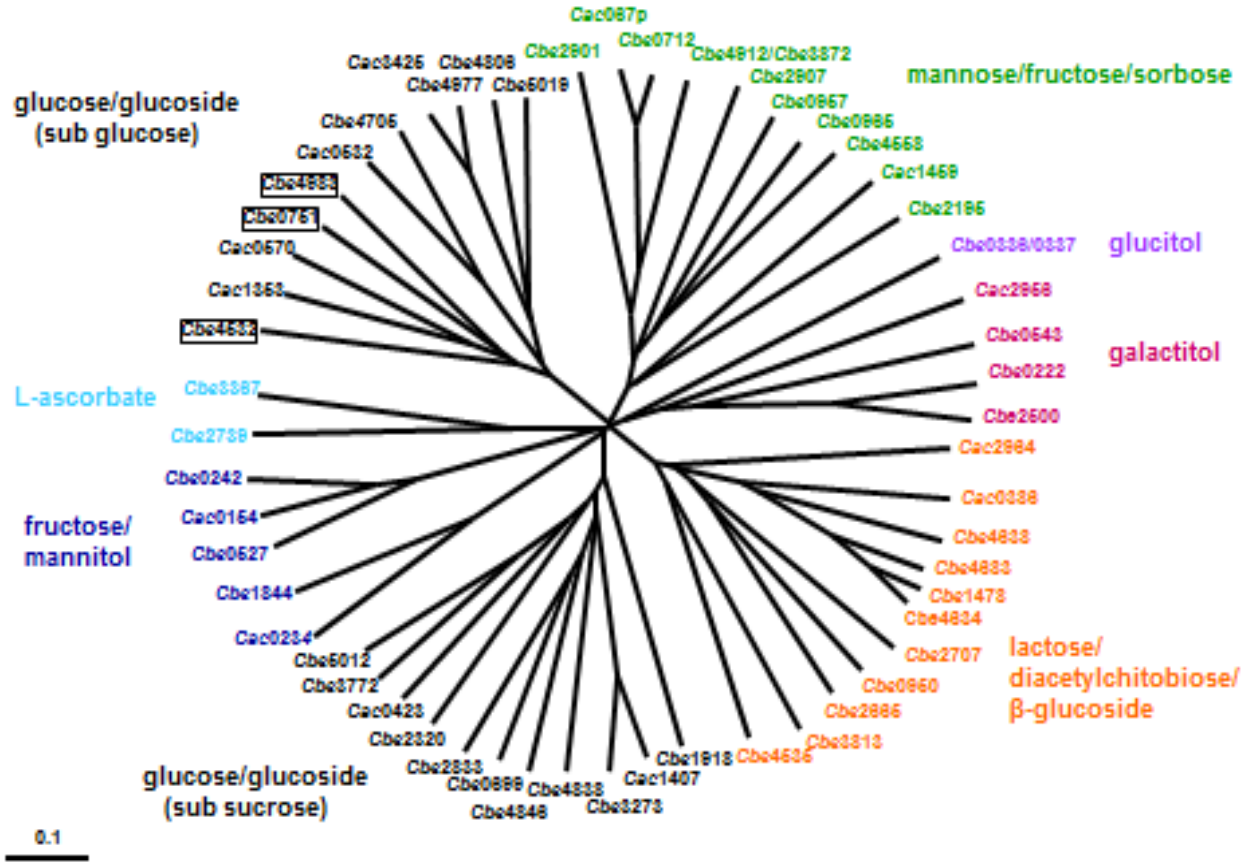


Figure 1.3 Phylogenetic tree of IIC domains of PTS of *Clostridium beijerinckii.* (Courtesy of W.J. Mitchell).

The tree shows different PTS families of *Clostridium beijerinckii* and their relationships. The systems are highlighted in colour according to the family to which they belong. The families shown are glucose/glucoside (black) divided into the glucose and sucrose sub-families, mannose/fructose/sorbose (green), glucitol (purple), galactitol (pink), lactose/diacetylchitobiose/β-glucoside (orange), fructose/mannitol (blue) and L-ascorbate (cyan).

1.10 The Mannose/Fructose/Sorbose Family:

While the mannose/fructose/sorbose PTS is structured similarly to other systems, it has some unique aspects. The most noticeable difference is that it is the only family in which its members possess a IID domain. Another is that while most systems are specific to either one or a few sugars, those belonging to the man/fru/sor family display a much broader specificity for a range of sugars. One example of this is the mannose porter found

in *E. coli* which has been studied in particular detail. It can transport and phosphorylate not just mannose, but also glucose, fructose, glucosamine, N-acetylglucosamine and N-acetylmannosamine (Plumbridge and Vimr, 1999).

The IIA ^{Man} domains of *E. coli* contain a doubly wound α/β superfold within their structure. The IIB ^{Man} domain also contains a doubly wound α/β superfold and only differs from the appearance of IIA ^{Man} in that it far more greatly resembles the protein phosphoglyceromutase (PGM). This is unsurprising since both IIB ^{Man} and PGM are used to catalyse phosphoryl transfer via a phosphohistidine and so possesses similar active site residues. The IIC ^{Man} proteins of *E. coli* are presumed to comprise either all or at least most of the sugar transporting channel. The IIC ^{Man} proteins contain six established transmembrane α-helical spanners, whereas IID ^{Man} possesses only one and has most of its polypeptide chain within the periplasm of the cell. Nevertheless, proteins IIC ^{Man} and IID ^{Man} are required for the transport of sugars into the cell (Gschwind *et al*, 1997).

1.11 Carbon Catabolite Repression:

Carbon catabolite repression (CCR) is a regulatory mechanism found in bacteria and some other organisms. Available carbon sources are utilised in a sequential order depending on the organism's preference. In most bacteria, glucose is preferred over all other carbohydrates. If more than one substrate is available, the presence of the preferred carbon source will inhibit the expression and activity of PTSs and other catabolic systems which allow the use of other carbon sources, until the preferred source has been utilised (Görke & Stülke, 2008). Once the sources of the preferred sugar begin to deplete, the PTS responsible for the uptake of the second most favourable sugar will be activated until that too is depleted and so forth. The uptake of a less favourable sugar is therefore repressed until there is no longer a more favourable option available. Carbon catabolite repression allows bacteria to optimise their growth rates in their natural environments by providing them with a complex mixture of nutrients (Stülke and Hillen, 1999).

As genes or operons which express catabolic systems for carbohydrates are almost exclusively regulated by specific control proteins which require inducers for a high level of expression, the system can be successfully controlled by either inhibiting or allowing the formation of inducers. The use of CCR allows bacteria to adjust to their physiological

condition and can prevent the metabolism of the organism from becoming overtaxed (Brückner and Titgemeyer, 2002). This type of regulation relies on different regulatory mechanisms in different species of bacteria. This includes transcription activation or repression by an RNA-binding protein. While CCR specifically relates to catabolism, it is not limited to repressing the expression of catabolic systems. It can also indirectly affect other related mechanisms such as the repression of virulence factors in pathogenic bacteria (Brückner and Titgemeyer, 2002).

In the majority of bacteria, enzymes involved in the transportation and phosphorylation of sugars including the PTS play an important role in signal generation, leading to CCR through various transduction mechanisms (Stülke and Hillen, 1999). In Gram positive bacteria, the phosphocarrier HPr is an important component of the CCR mechanism. As mentioned previously, HPr in Gram positive bacteria has two sites that can be phosphorylated; a histidine (His-15) and a serine (Ser-46). PEP is used to phosphorylate EI, which in turn passes the phosphate to the His-15 residue and thence via EIIA and EIIB to the transported sugar. On the other hand, protein kinase HPrK transfers a phosphate from ATP to the Ser-46 residue of HPr. The phosphorylated HPr then binds to the catabolite control protein CcpA, resulting in binding to DNA and repression of target genes (Figure 1.4). The link to glucose uptake is provided through stimulation of HPrK activity by fructose-1.6-biphosphate that is generated as a result of glucose metabolism. The CcpA protein plays a crucial role in the regulation of metabolic pathways in many Gram positive bacteria and is a member of the LacI/GalR transcription regulator family (Lyer et *al*, 2005, Deutscher, 2008). Its role is central in the CCR of low G-C firmicutes, including endospore forming species (Singh et *al*, 2008). Not only is it involved in the regulation of metabolism in clostridia, it is also involved in the regulation of sporulation and toxin production in some pathogenic species (Varga et *al*, 2008, Varga et *al*, 2004).

In clostridia, CCR evidently plays an important role in the utilisation of sugars and appears to be related to PTS function (Deutscher et *al*, 2006). While *C. beijerinckii* has shown a preference for glucose over other sugars (Mitchell, 1996; Tangney et *al*, 1998), there have been no other detailed studies of CCR in this particular species. CcpA also plays a key role as a repressor of D-xylose and L-arabinose metabolism in *C. acetobutylicum* (Ren et *al*, 2012), as shown using DNA microarrays and then phenotypic, genomic and biochemical validation.

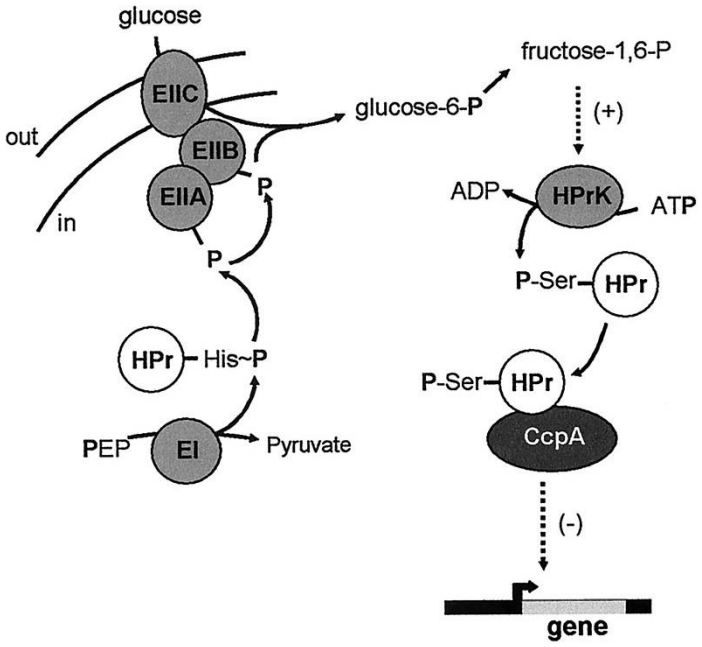


Figure 1.4 Mechanism of CcpA-dependent CCR in Gram-positive bacteria (courtesy of Warner and Lolkema, 2003).

In order to fully understand and manipulate sugar utilisation in clostridia, it is important that each group of PT-systems is appropriately categorised in regard to their regulatory and catalytic functions.

1.12 Biofuel Production from Waste Materials:

The reason why fermentative butanol production has largely ceased is due to the fact that it is no longer economically viable compared to the low cost of converting fossil fuels. One way in which production costs can be lowered to increase the sustainability of the process is to use waste materials as a carbohydrate source for the bacteria.

Waste is defined as any unwanted material or substrate which will potentially cause changes in the ecosystem (Al-Makishah, 2013). The term is most frequently used to describe byproducts produced by factories and households (acetone, soybean starch, other used and unwanted items) If not disposed of properly, wastes can cause harm to the environment and to wildlife.

As waste is unwanted by the company that produces it, organic waste is far cheaper to gather than purified materials such as starch or sugar. The disadvantages to using an unpurified carbohydrate are firstly that ABE production is less likely to be as consistent as when using pure materials and the quality of the final product may therefore vary and secondly that there will likely be indigestible residue left after each batch and so equipment would require frequent cleaning. It may be possible to at least partially purify waste products, which whilst making the end products less variable would increase the overall cost and time of ABE production. In any case, it is desirable to have a complete understanding of the metabolic capabilities of the fermenting bacterium so that utilisation of waste materials can be optimised.

1.13 Aims of the Project:

The metabolic diversity of *C. beijerinckii* in terms of sugar uptake is indicated by gene analyses showing similarities between the sequences of its PTSs and those from other species which have already been fully characterised. Due to its large number of PTSs belonging to various substrate families, *C. beijerinckii* is of great interest as it is potentially able to produce solvents from a wide range of materials. In order to fully utilise these abilities of *C. beijerinckii*, its routes for sugar uptake, metabolism and their regulation need to be described and fully characterised. The mannose/fructose/sorbose family of PTSs is of particular interest due to the fact that mannose is found in a large variety of plant waste materials including corn stover and certain types of lignocellulosic biomass.

This project aims to gain further understanding of the mannose/fructose/sorbose family of PTSs in *C. beijerinckii* NCIMB 8052 by identifying which PTSs have the ability to transport mannose. This will be done by amplifying genes associated with the PTS domains and inserting them into a strain which is unable to transport mannose. Transformed cells will then be tested for their ability to utilize mannose.

2. Materials and Methods

2.1 Sources of Bacterial Strains

Escherichia coli SoloPack competent cells were obtained from Agilent Technologies. The *E.coli* mutant ZSC113 (genotype; gpt-2-mpt-2 glk-7 *AstrA*) used for determining whether or not a plasmid possessed the correct genetic information to allow a cell to use mannose and glucose was isolated by Curtis and Epstein (Curtis and Epstein, 1975) and obtained from the Coli Genetic Stock Center. ZSC113 was taken from storage at -70^0C and aseptically transferred onto Luria agar (LA) plates containing no antibiotic. Once streaked with the sample, the plates were incubated at 37^0C overnight. Once colonies appeared on a plate, one colony was aseptically transferred to 10ml of Luria-Bertani broth and again, incubated at 37^0C overnight to provide a working culture. The genes for phosphotransferase systems cloned into *E.coli* were already purified from *Clostridium beijerinckii* NCIMB 8052. This strain had been obtained from the National Collection of Industrial and Marine Bacteria (NCIMB).

2.2 Materials Used:

2.2.1 Media

Two different types of media were used for growing bacterial cultures during the investigation. Both media were sterilised by autoclaving at 121^0C for 15 minutes.

Luria-Bertani (LB) medium was made consisting of 10g tryptone, 5g yeast extract and 5g NaCl per litre of distilled water. Technical Agar no3 (Oxoid) was added to a concentration of 12g/l to prepare Luria agar when required. Ampicillin was added to some broths/ plates at 100µg per ml of broth/agar. Kanamycin was added to some broths/plates at 30µg per ml of broth/agar. Antibiotics were not added to media until it had cooled to a minimum of 55^0C to ensure that they were not degraded by heat.

Initially, MacConkey medium was prepared consisting of 17g of Bacteriological peptone (Oxoid), 3g proteose-peptone (Oxoid), 1.5g bile salts (Difco), 5g NaCl, 0.075g neutral red, 0.2 ml of 0.5% crystal violet solution and 1% glucose or mannose per litre of distilled water. Technical Agar no3 (Oxoid) was added at12 g/l to prepare agar when required. Thereafter, MacConkey medium was made using MacConkey Agar Base (MCAB) (Difco) with sugar added to a concentration of 1% of 10g per L of Technical Agar no3. Technical Agar no3 had to be added to MCAB plates containing mannose, because it was found that they would not set without it. Antibiotics were added as required.

2.2.2 Buffers and Solutions

A description of each of the buffers and solutions used in the project and their ingredients can be found in Appendix IV.

2.3 Cloning of Phosphotransferase System Genes

2.3.1 Primers

Primers were designed for the amplification of genes encoding domains of the selected PTSs of *Clostridium beijerinckii* NCBIMB 8052. Primers were also designed for genes encoding the mannose PTS of *Escherichia coli* ZSC113. The primers were designed to consist of approximately 20 nucleotides in order to make them as specific as possible whilst keeping the annealing temperature in a suitable range (typically 35^0C to 60^0C). The melting temperature of each primer was calculated according to the base composition as [2x(A+T) + 4x(G+C). If PCR produced no product when a pair of primers was first used, the next step was to gradually lower the annealing temperature until signs of product appeared. A full list of the primers used and their sequences can be found in Table 2.1. The pairs of primers and their corresponding melting temperatures can be found in Table 2.2. The pairs of primers that were used and their corresponding melting temperatures can be found in Table 2.3.

All primers used were purchased from MWG Eurofins. Molecular grade water was added to produce a concentration of 100mol/µl and primers were then stored in the freezer at -20^0C. Primer positions relative to the amplified genes can be found in Appendix I.

Table 2.1 Primers used over the course of the investigation.

Primer Name	Reference No.	Primer Sequence
cbei 0711 F	396	5'-GGTGTACAAATATTGTGACG-3'
cbei 0712 F	397	5'-ATTCAAAAGCAAATATGGACG-3'
cbei 0713 R	398	5'-ACATGCTTTTATCAATTATTCC-3'
cbei 3874 F	700	5'-CAGAAAAGGTAGTTATAGCC-3'
*cbe*i 3872 F	701	5'-TTAGAGGTAAGGAAACTAGC-3'
cbei 3871 R	702	5'-TAATACAAGTATAGACTACCC-3'
cbei 0955 F	703	5'-TCCTCACTAAAATAGGTCAAC-3'
*cbe*i 0957 F	704	5'-GAGATAAGACAGGTTCCAGG-3'
cbei 0958 R	705	5'-TGCATCTCTAATATTAAGCTGC-3'
cbei 0963 F	706	5'-ATGATTTCAAGCGATTAAGAG-3'
c*bei* 0965 F	707	5'-CAGATTTACTAAAATAAGTAAGG-3'
cbei 0966 R	708	5'-TCATTTTTATTACCTCTTTCTC-3'
E.coli Man F	712	5'-CTGAATCGATTCGATTGTGG-3'
E.coli Man R	713	5'-AAAGAATCAGTACCAGGTCC-3'
M13 R	372	5'-CAGGAAACAGCTATGACC-3'
T7 Promoter	373	5'TAATACGACTCACTATAGGG-3'

Primers used for cloning that were used throughout the investigation are listed above in the table with their corresponding sequences. *Cbei* numbers refer to locus numbers in the *C.beijerinckii* genome.

Table 2.2 Primers used for Sequencing

Primer Name	Primer Annealing Sequence
Forward Sequencing Primer	5'-CTGAATCGATTCGATTGTGG-3'
Reverse Sequencing Primer	5'-GGACCTGGTACTGATTCTTT-3'

Primers used for sequencing the *manXYZ* genes of *Escherichia coli* ZSC113. The exact locations of the annealing sites within the genes are shown in Figure 5.6 of Appendix II.

Table 2.3 Primers used and their melting temperatures

Genes Included	Primer Pairs	Calc Temp (^{0}C)	Opt Temp (^{0}C)
*cbei*0712-0713	397+398	56	55
cbei3871-3874	700+702	56	56
*cbei*3871-3872	701+702	56	48
*cbei*0955-0958	703+705	58	53
*cbei*0957-0958	704+705	60	48
*cbei*0963-0966	706+708	56	36
*cbei*0965-0966	707+708	58/56	36
E. coli (fwd+rev)	712+713	58	35

All primers that were used throughout the investigation are listed above in the table, with the exceptions of M13 Reverse Primer and T7 Promoter Primer. These primers were paired with various other primers during the screening stage and so their optimal temperatures varied depending on the lower of the two melting temperatures. *Cbei* numbers refer to locus numbers in the *C.beijerinckii* genome. Primer Pairs refers to the two primers used per PCR. Calc Temp= calculated melting temperature. Opt Annealing Temp= Melting temperature which gave optimal results in PCR.

2.3.2 Polymerase Chain Reaction (PCR)

Primers were used in pairs as appropriate to amplify the required genes. When a product suitable for cloning was required; 10µl of prepared 5x reaction mix (ingredients listed in Appendix V) was added to 36.5µl of molecular grade water, with 1µl of each primer and of 1µl DNA template. After an initial 5 minutes at 95^{0}C, 0.5µl of DNA polymerase was added giving a final volume of 50µl before allowing the reaction to continue. The DNA polymerase used was Easy-A® High-Fidelity PCR Cloning Enzyme (Agilent Technologies). In some later reactions, 2x Master Mix which included the Easy-A Enzyme replaced the reaction mix and pure Easy-A Enzyme. When the Master Mix was used, the composition of the reaction was 1µl of each primer, 1µl of template DNA, 22µl of molecular grade water and 25µl buffer (Master Mix).

For screening purposes; the PCR amplification was carried out using 10µl of 2x Biomix (Bioline) with 7µl of molecular grade water and 1µl of each primer (forward and reverse) and 1µl of DNA template, giving a final volume of 20µl.

Samples were amplified according to the following protocol:

Stage 1 was set to 95^0C for 5 minutes. In stage 2 the sample was heated at 95^0C, then cooled to an appropriate annealing temperature and finally increased to 72^0C. Each of these temperatures was held for 30 seconds. The cycle in stage 2 was repeated 35 times to allow the sequence to be amplified. Stage 3 heated the sample to 72^0C for 7 minutes before lowering the temperature to 4^0C until the sample was collected from the thermal cycler and stored in the fridge.

2.3.3 Agarose Gel Electrophoresis

DNA from PCR and plasmid samples was examined using gel electrophoresis. The agarose gel was made by mixing agarose (Helena BioSciences) at 1% w/v with 1×TAE buffer and microwaving the mix until it boiled. Once the agarose mixture had been left to cool for approximately 5 minutes, 1µl of GelRed (Merck) was added per 10ml of mixture. The agarose was swirled to distribute the GelRed throughout the mixture and then poured into the tray. A comb was situated at one end of the gel to form wells where samples could then be placed. Once solidified, the gels were transferred to the electrophoresis tank and immersed in 1×TAE buffer.

Samples of the DNA were prepared by adding 3µl of molecular grade water and 1µl of 5×DNA loading buffer to 1µl of PCR product/plasmid. 5µl of Hyperladder I (Bioline; see Appendix V) was added to a well in the agarose gel and 5µl of each sample were loaded into separate neighboring wells. The machine was then set to 90 volts, 400 Amp for 35 minutes. After running, the gels were viewed and photographed using a BioRad ChemiDoc XRS+ Molecular Imager.

2.4 Cloning the genes

The PTS genes that had been successfully amplified by PCR were then cloned and transformed into *E.coli* SoloPack competent cells using the StrataClone Cloning Kit (Agilent Technologies). The plasmid 'PCR Cloning Vector pSC-A-amp/kan' (Appendix III) was used which, as its name suggests, contains genes for resistance to both ampicillin and kanamycin for selective purposes.

The steps described in the StrataClone PCR Cloning Kit Manual were followed precisely. The ligation reaction was prepared by adding 3µl of StrataClone Cloning Buffer, 1µl of PCR product in a 1:10 dilution and 1µl of StrataClone Vector Mix in that order. The mix was incubated for 5 minutes at room temperature before adding 1µl of the reaction to a thawed tube of StrataClone SoloPack competent cells. Once added, the competent cells were incubated on ice for 20 minutes. After incubation, the transformation mixture was heat shocked for 45 seconds at 42^0C. The transformation mixture was then stored on ice for another two minutes before 250µl of LB was added and the cells were left to recover at 37^0C for at least one hour. After recovery, the transformed cells were plated onto LA containing 100 µg/ml ampicillin which had been spread with 40µl of 2% X-gal. The X-gal was used to screen for colonies which did not contain recombinant vector since they would appear blue in colour. The LA plates were incubated at 37^0C overnight before colonies could be viewed.

2.5. Screening for the Presence of the Cloned Insert

After the cloning process, the transformed StrataClone *E. coli* cells were gown on selective LB plates containing either ampicillin or kanamycin with the addition of X-gal. Colonies that were white or light blue in colour were then picked using a sterile toothpick, numbered and patched onto a fresh plate of the same description. A dark blue colony was also patched to be used as a negative control. A sample was taken from each patched colony and mixed into 20 µl of molecular grade water in a 50µl PCR tube. The PCR tube was heated at 100 ^{0}C for 10 minutes. The samples were then centrifuged at 6,000 rpm for 3 minutes and then stored on ice. A fresh PCR tube was filled with 7µl of molecular grade water, 10µl of 2x Biomix from Bioline, 1µl of each relevant primer (2 µl together) and 1µl

of the heated cell sample to be used as the template. A PCR was then carried out as described above in 2.3. Once the PCR was complete, the sample was examined using electrophoresis. If a band of the correct size appeared, it was assumed that the sample contained the cloned insert.

2.6 Determining the Orientation of DNA Inserts

The orientation of the cloned insert in each plasmid was determined so see whether or not the gene had been inserted in the more favourable orientation for expression i.e. under control of the *lac* promoter in the vector (Appendix III). In order to achieve this, further PCR reactions were carried out, but using different sets of primers. One cloning primer, either the forward or reverse, was added to the reaction mix with the addition of either the T7 promoter primer or an M13-Rev primer. The T7 and M13-Rev primers anneal to the cloning vector a short distance from where the inserted gene sequence is placed. Depending on which primer combinations produced a product, the orientation of the inserted gene could be determined.

2.7 Plasmid Preparations

2.7.1 Small-scale preparation of plasmids using the miniprep kit

The Fermentas GeneJET^{TM} Plasmid MiniPrep Kit (#K0502) was used to purify plasmids from colonies which had been successfully transformed during the cloning process. The procedure for obtaining purified plasmid using the kit was as follows:

A starter culture incubated with a colony which was shown to contain the insert was incubated at 37^0C overnight in10ml of LB containing 100µg/ml ampicillin.

The cells were then harvested by collecting 5ml of culture in a microcentrifuge tube and centrifuging it at 6,000 rpm for 5 minutes. The 5ml of culture was microfuged 1ml at a time, the supernatant being discarded after each centrifuge spin and the cells collected in a cumulative pellet. The final supernatant was disposed of and the cells were resuspended in 250µl of Resuspension Solution by vortexing. 250µl of Lysis Solution was then added and

mixed by inverting the tube 6 times. Once the solution became viscous and clear, 350µl of the Neutralization Solution was added and mixed by inverting the tube 6 times until the mixture formed white clumps of cell debris. The mixture was then centrifuged again at 6,000 rpm for 5 minutes so that the debris would form a pellet. The supernatant was transferred to a GeneJET spin column (provided with the kit) without disturbing the pellet. The mixture was then centrifuged for 1 minute so that the filter in the GeneJET column would retain plasmid DNA, separating it from the rest of the solution. The flow-through was discarded and 500µl of Wash Solution was added. The mixture was then centrifuged again for 1 minute, the flow-through disposed of and another 500µl of Wash solution was added. The mixture was centrifuged for 2 minutes before the top of the GeneJET column was transferred to a fresh microcentrifuge tube. 50µl of Elution Buffer was added to the centre of the GeneJET column membrane whist taking care that the pipette tip did not make contact with the membrane itself. The mixture was then incubated at room temperature for 2 minutes before being centrifuged for 2 minutes. This transferred the plasmid DNA through the membrane and into the microcentrifuge tube. The solution containing the purified plasmid was then stored at -20^{0}C for later use.

2.7.2 Large-scale preparation of plasmids using the midiprep kit

For larger scale plasmid preps, the GeneJET Midiprep Kit (#K0481) was used. In order to prepare the bacterial culture, a single colony was picked from a freshly streaked plate containing either ampicillin or kanamycin and inoculated into 5ml of LB broth, containing the same antibiotic. The culture was incubated at 37^{0}C for 8 hours in a shaker at 250 rpm. After the 8 hour growth period, the culture was diluted by adding it to 50ml of fresh LB with kanamycin or ampicillin. It was then left to incubate in the same conditions for 16 hours overnight. Once the culture had reached the optimal optical density for plasmid DNA isolation (OD_{600}= 2-3) the maximum volume of culture to be used was calculated using the equation: (V)ml = 150/OD_{600}, up to 50 ml of culture was transferred to a sterile 50ml centrifugation tube. The cells were then collected by centrifugation at 5,000xg for 10 minutes and the supernatant was discarded

.

The pellet of bacterial cells was resuspended in 2ml of Resuspension Solution by vortexing. Once the cells were fully suspended, 2ml of Lysis Solution was added and mixed by inverting the tube 6 times and was then incubated at room temperature for 3

minutes. After the brief incubation period, 2 ml of Neutralization Solution was added and mixed by inverting the tube 8 times. The next step was to add 0.5 of Endotoxin Binding Reagent, mixing again by inverting the tube 8 times before allowing the mixture to incubate for 5 minutes at room temperature. Once mixed with Neutralization Solution and Endotoxin Binding Reagent, the solution formed cloudy white clumps of cell debris. To further separate the free DNA in the solution from the cell debris, the tube was centrifuged for 20 minutes at 18,000xg. The solution was then transferred into a 15ml tube using a pipette, with care being taken not to disturb the pellet of cell debris. One volume of 96% ethanol was added and mixed by inverting the tube 6 times. Part of the sample (5.5ml) was transferred to a collection tube containing a column, supplied within the kit, and centrifuged for 3 minutes at 2,000xg. The flow-through was discarded and the column replaced into the collection tube. Any remaining sample (up to 5.5ml) was transferred to the collection tube and centrifuged under the same conditions. Next, 4ml of Wash Solution I was added to the column and centrifuged for 2 minutes at 3,000xg. Once the flow-through was discarded, 4ml of Wash Solution II was added and the tube was centrifuged again for 2 minutes at 3,000xg. The sample was then centrifuged for an additional 5 minutes at 3,000xg to remove any residual Wash Solution. Once the Wash Solution was removed, the plasmid was eluted from the column using 50µl of Elution Buffer and moved to an Eppendorf tube for storage. The purified plasmid was then stored at -20^0C until later use.

2.8 Ensuring the Orientation of DNA Inserts

The orientation screening procedure described in section 2.6.1 was repeated using the pure plasmid to ensure that the cloned insert was indeed in the optimal orientation before proceeding to the next stage.

2.9 Transformation of Plasmid into *E.coli* ZSC113

To transform a plasmid into the *E. coli* ZSC113 mutant, the following steps were taken:

A culture was prepared by taking a single colony of the mutant from an LA plate and adding it to10ml of LB, then incubating at 37^0C overnight. A sample of 1ml was taken from the overnight culture and added to a flask containing 100ml of LB. This flask was then left to incubate at 37^0C for a period of 3-5 hours until the OD_{600} had reached 0.4 to 0.6. The next step was to transfer 50ml of the culture to a sterile polypropylene tube which was then stored on ice for 10 minutes. Cells were then recovered by centrifuging the sample at 4,000g at 4^0C for 10 minutes. After centrifugation, the supernatant was disposed of and the cells were resuspended in 10 ml of ice cold 0.1 M $CaCl_2$ solution. The sample was then centrifuged under the same conditions as previously. Again, the supernatant was disposed of and the cells were resuspended in 2ml of the $CaCl_2$ solution and stored on ice.

A 200µl sample of the cells was transferred to a sterile Eppendorf tube and 1µl of plasmid was added and gently mixed by inverting the tube 4-6 times. The mixture was then stored on ice for 30 minutes. After the incubation period, the samples were heated in a water bath at 42^0C for 90 seconds and returned to the ice for 2 minutes. 800µl of LB medium was mixed into the sample which was then incubated at 37^0C for at least 1 hour. 50µl and 100µl of the sample was then spread onto LB agar plates containing 100µg/ml ampicillin or 30µg/ml kanamycin and incubated at 37^0C overnight.

2.10 Analysis of mannose and glucose utilisation by cells

2.10.1 Sugar fermentation on MacConkey agar

Colonies that grew on the LB amp or LB kan (ampicillin added at 100µg/ml, kanamycin 30µg/ml) plates after the transformation procedure were restreaked onto a fresh LB amp or LB kan plate and left to grow overnight at 37^0C. Each colony was then streaked onto a MacConkey agar plate containing ampicillin or kanamycin and either glucose or mannose as a fermentable carbon source. After overnight incubation at 37^0C, the colour of the colonies and surrounding agar was noted. Colonies that were pale and had turned the surrounding agar to an orange/yellow colour could not ferment the added sugar (negative result), whereas colonies that had turned a red/pink/purple colour could ferment the added carbon source (positive result).

2.10.2 Utilisation of sugar during growth in LB medium

A sample of the transformed *E.coli* ZSC113 was added to 10 ml of LB broth with added ampicillin (100µg/ml) or kanamycin (30µg/ml) and either glucose or mannose at a concentration of 1%. The culture was incubated overnight at 37^0C while shaking at 250 rpm. After incubation, 1 ml of culture was transferred to a 250 ml conical flask containing 100ml of LB plus the same antibiotic and carbon source as previously added, and incubated again at 37^0C. This time, 25mM of glucose or mannose was added to the LB. The initial OD_{600} was measured before incubation and then measured again hourly for 8-9 hours. Samples of the culture (0.5ml) were placed in Eppendorf tubes at each interval at which the OD_{600} was recorded. These samples were centrifuged for 10 minutes at 13,000 rpm and the supernatant transferred to a fresh Eppendorf tube. These samples were then stored at -20^0C until the sugar concentration could be measured. This was done using high performance liquid chromatography (HPLC). The columns used were a Dionex Carbopac PA-1 Guard column, 4 x 50mm and Dionex Carbopac PA-100 column, 4 x 250mm, and the detection was by a pulsed amperometric detector (PAD).

As a control, a sample of *E .coli* ZSC113 cells was transferred into 10ml of LB broth without antibiotic and incubated overnight with shaking at 37°C. 1ml taken from the overnight culture was transferred to a 250 ml conical flask containing 100ml of LB Broth with 50µg/ml ampicillin and 25mM glucose or mannose and incubated at 37ºC. Growth

was measured by following $OD_{600.}$ Culture samples (0.5ml) were taken every hour, transferred to an Eppendorf tube, and then centrifuged at 13,000 rpm for 10 minutes. The supernatants were removed and transferred to fresh tubes and stored at -20ºC for the measurement of sugar concentration, by high performance liquid chromatography (HPLC).

2.11 DNA Sequencing

The *man*XYZ genes of *E. coli* ZSC113 that encode the mannose PTS were isolated to see where the mutation(s) that rendered the system non-functional lie. The primers 712 and 713 were used to amplify the full gene sequence of the *manXYZ* genes and the amplified strands were inserted into the vector as for the other gene systems. A large amount of plasmid was collected using the midi kit. The plasmid solution was diluted to 50ng/µl and sent to Beckman Coulter Genomics for sequencing by primer extension. The primers 372 and 373 which anneal to the vector were used initially. Sequence obtained from the first reaction from each end of the cloned PCR product was used as a primer to synthesise the next strand of DNA which would likewise be sequenced by chain termination. The primers 372 and 373 which anneal to each end of the vector were used to design a new primer so that the next region could be sequenced, and so on until the entire sequence was obtained on both strands (Fig. 2.2). Three sequence reactions in each direction were required, in order to obtain the entire sequence.

2.12 Bioinformatics:

The genes that encode each domain of all PTSs of *C. beijerinckii* NCIMB 8052 studied were collected from the NCBI website (http://www.ncbi.nlm.nih.gov/). Genes encoding the PTSs shown to belong to the mannose-fructose-sorbose family from several other species of bacteria were found on the Transporter Classification Database (http://www.tcdb.org/superfamily.php). As several nucleotide codons can encode the same amino acid, it was decided to analyse the data in the form of amino acid sequences. This way a phylogenetic tree would show the differences in genes based on the function of the protein and not solely on base sequences. Identity percentage between pairs of proteins was obtained using the BLAST program on the NCBI website. Multiple sequences were aligned using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) in order to create a base for the phylogenetic tree in Newick format. The final phylogenetic tree was made using the code in Newick format in Java TreeView.

3. Results

3.1 Bioinformatics:

In order to establish whether there is a relationship between the nine mannose-fructose-sorbose family phosphotransferases of *Clostridium beijerinckii* and the PTSs of other species which are known to transport mannose, the amino acid sequences of their IIC domains were compared. The amino acid sequences were chosen rather than the nucleic acid sequences so that the end function of the proteins could be properly compared, as different combinations of nucleic acids can encode the same amino acid. Also, since the IIC domain interacts with the substrate, relationships are more likely to reflect substrate specificity. Several protein BLAST analyses were carried out and the sequences were then aligned using Clustal Omega. As shown in the phylogenetic tree (Figure 3.1) the majority of PTSs analysed which belong to *C. beijerinckii* (Cbei2196, Cbei2907, Cbei0957, Cbei0965 and Cbei2901) are on the same branch and are shown to be most closely related to the putative hexose PTS of *Myxococcus xanthus* and ManLNM of *Listeria monocytogenes* (all in green). Cbei3872 and Cbei4912 are almost identical and most closely relate to the fructose system of *Bacillus subtilis* and to a lesser extent to the sorbose transporter of *Klebsiella pneumonie* and the mannose system of *Escherichia coli* (all in purple). Cbei4558 was not closely related to the other transporters of *C. beijerinckii* and instead showed a greater similarity to the fructose transporter of *Streptococcus pneumoniae*, the fucosyl-α-1,3-N-acetylglucosamine transporter of *Lactobacillus casei*, the glucoselysine/fructoselysine transporter of *Salmonella typhimurium* and the gluconate transporter of *Enterococcus faecalis* (all shown in blue). Similarly, Cbei0712 more closely resembles IIC proteins of systems from other species rather than the other systems from *C. beijerinckii*. It showed the closest similarity to ManLMN of *Lactobacillus casei* and shared more in common with the hexose (fructose and glucose demonstrated) system of *Oenococcus oeni*, the GluMan system of *Listeria monocytogenes*, the mannose system of *Streptococcus thermophilus* and the mannose transporter of *Lactococcus lactis* (all shown in red).

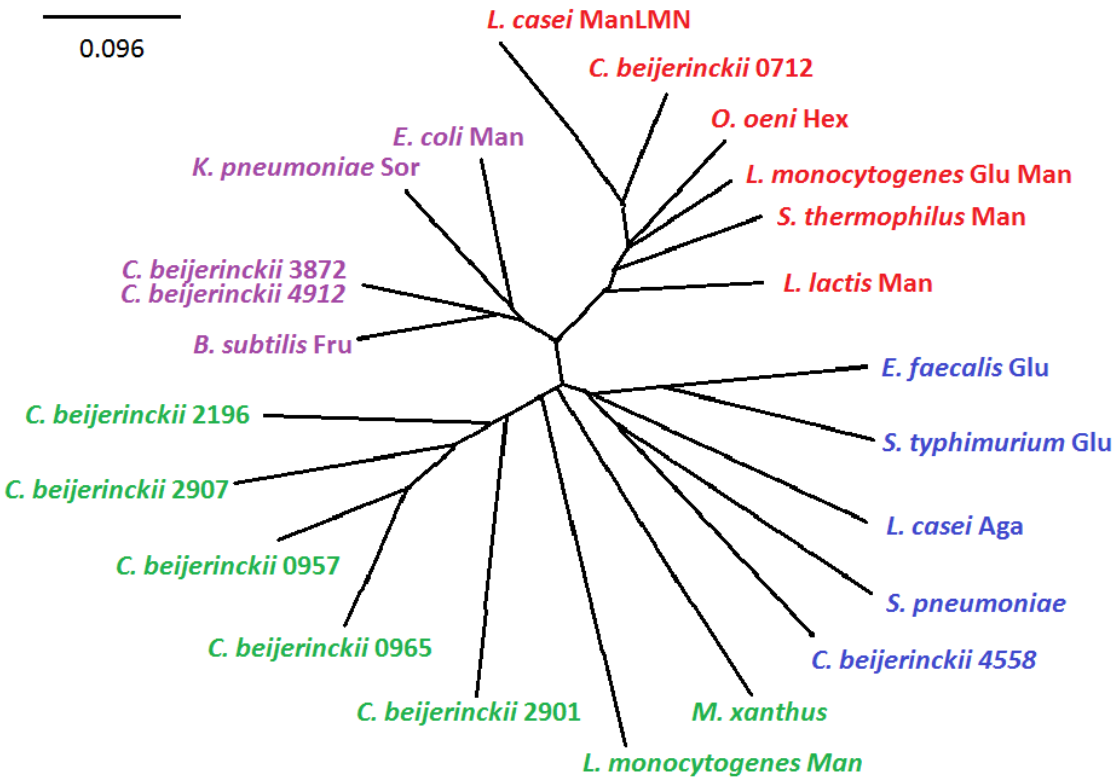


Figure 3.1. Phylogenetic tree of PTS Enzyme IIC domains belonging to the Man-Fru-Sor Family

The tree shows different IIC domains of the PTSs belonging to the mannose-fructose-sorbose family in several species of bacteria. Among them are the 9 systems of that family belonging to *Clostridium beijerinckii* and the mannose PTS of *Escherichia coli*. The systems are highlighted in colour according to apparent phylogenetic groups.

The BLAST results of the IIC domains of the four PTSs of *Clostridium beijerinckii* studied in this investigation compared to each of the IIC domains of other PTSs known to belong to the mannose-fructose-sorbose subfamily are shown in Table 3.1.

Table 3.1: BLAST results showing identity for deduced amino acid sequences of the IIC domains of mannose-fructose-sorbose family PTSs compared with the IIC domains Cbei0712 and Cbei 3872 of *C. beijerinckii*. NSF= No significant identity found.

Species	Cbei 0712 % identity	Cbei 3872 % identity	Cbei 0957 % identity	Cbei 0965 % identity
B. subtilis Fru	47	71	33	60
K. pneumonie Sor	48	61	29	56
L. casei ManLMN	77	47	78	NSF
L. lactis Man	56	48	28	28
O. oeni Hex	67	51	38	50
E. coli Man	53	64	43	40
S. thermophilus Man	58	45	60	47
E. faecalis	29	24	29	29
S. pneumoniae	26	33	34	23
M. xanthus	32	38	35	25
L. casei Aga	27	31	29	44
L. monocytogenes Glu Man	74	47	NSF	37
L. monocytogenes Man	NSF	NSF	NSF	NSF
S. typhimurium Glu	27	30	26	30

A comparison was also made between the amino acid sequences of the Enzyme IIAB domains of the four *C. beijerinckii* PTSs and of those of other species also belonging to the mannose-fructose-sorbose family. Since the majority of IIA and IIB domains in the non-*C.beijerinckii* systems were combined as one single protein, the IIA and IIB domains of *C. beijerinckii* were combined so that a better comparison could be made. The results from the BLAST analyses are shown in Table 3.2 below. In the case of Cbei0710-0711, the closest relationship was found to be to the ManReg IIAB domain of *L. monocytogenes* (48% identity) and the least similarity to the IIAB of the ManLMN PTS of *L. casei* (no significant identity found). While comparing Cbei3873-3874, it was found that the greatest similarity was also to the IIAB domains of the ManReg system of *L. monocytogenes* and the least amount of similarity to the DgluSam PTS of *S. typhimurium* (23% identity). Cbei0955-0956 were found to have the closest relationship to the hexose PTS *of O. oeni* (28 % identity) and the least to the DGluSam PTS of *S. typhimurium* (19% identity). Finally, Cbei0963-0964 had the greatest similarity to the ManReg PTS of *L. monocytogenes* (31% identity) and the least similarity to the *L. casei* ManLMN PTS (23% identity). On average, the IIC domains are more closely related than the IIAB domains.

This may suggest that the evolution of the different domains must have occurred independently. It also indicates that the IIC domains are more likely to be responsible for substrate specificity rather than the IIB domains as suggested by Shi et al (2011).

Table 3.2: BLAST homology results for the deduced amino acid sequences of the IIAB domain of the mannose, fructose and sorbose subfamily PTSs compared with the IIAB domain Cbei0711 and Cbei3873-Cbei3874 of *C. beijerinckii.* NSF = No significance found.

Species	Cbei 0711 % identity	Cbei 3873-3874 % identity	Cbei 0955-0956 % identity	Cbei 0963-0964 % identity
B. subtilis Fru	24	40	25	28
K. pneumonie Sor	19	37	25	25
L. casei ManLMN	NSF	39	26	23
L. lactis Man	28	40	26	27
O. oeni Hex	41	38	28	26
E. coli Man	26	51	22	27
S. thermophilus Man	36	40	27	27
E. faecalis	26	30	26	27
S. pneumoniae	42	30	26	27
M. xanthus	36	27	23	27
L. casei Aga	31	31	27	28
L. monocytogenes Glu Man	25	44	25	24
L. monocytogenes Man	48	51	27	31
S. typhimurium Glu	26	23	19	24

3.2. Phosphotransferase systems Cbei 2901-2903 and Cbei 0711-0713 :

The phosphotransferase systems Cbei2901-2903 and Cbei0711-0713 were the first to be investigated. These two systems had previously been examined by Lennon (2009), but results regarding the functions of the systems were inconclusive. In particular, recombinant strains of the *E. coli* mutant ZSC113 containing Cbei0711-0713 had showed both positive and negative phenotypes on MacConkey agar plates. Because of this, it was decided to investigate these systems again in hope of clarifying whether they transport and phosphorylate mannose.

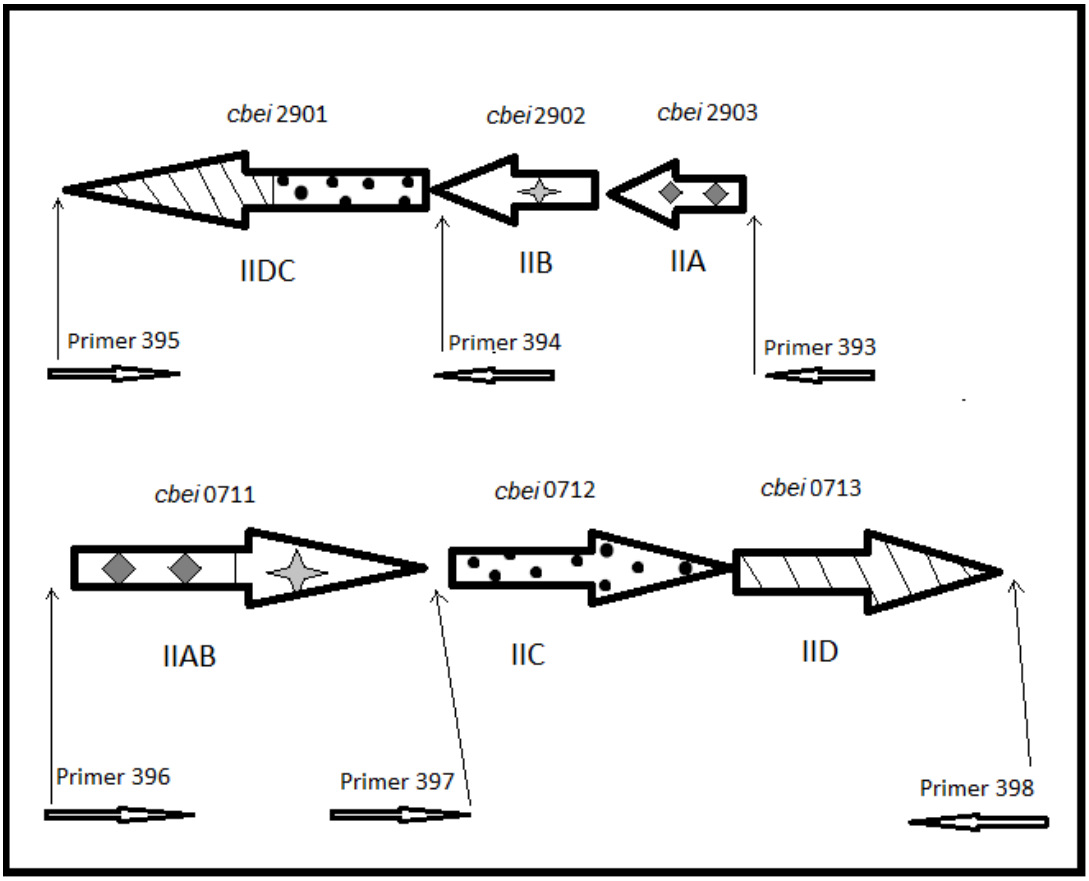


Figure 3.2. The arrangement of *Clostridium beijerinckii* NCIMB 8052 genes cbei2901 to cbei2903 and cbei0711 to cbei0713.

The large arrows represent the genes for the phosphotransferase system *cbei*0711-0713 of *Clostridium beijerinckii* and their directions in the genome. Smaller arrows indicate which primers were used to amplify certain genes. They also show where the primers would attach and whether they were in the forward or reverse orientation.

3.2.1 The amplification of PTS genes *cbei*2901-2903 and *cbei*0711-0713:

The genes for Cbei2901-2903 and Cbei0711-0713 were amplified by PCR using the primers shown in Figure 3.2. There were two pairs of primers used for each of the two systems. One set amplified the genes for all domains (IIA, IIB, IIC and IID) and the other amplified only the IIC and IID domains. Only one set of primers produced a product (397+398), which was of the expected size around 3,000 base pairs (2,750 bp) shown in Figure 3.3. Failure to obtain product from the other reactions may have been due to the fact that the primers used had been stored in the freezer for several years.

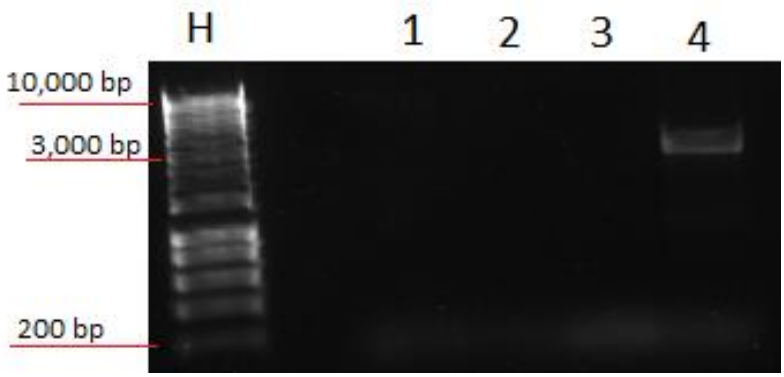


Figure 3.3. The amplification of genes *cbei*2901-2903 and *cbei*0711-0713

Four pairs of primers were used in an attempt to amplify two phosphotransferase systems from *Clostridium beijerinckii* NCIMB 8052 (395+393 = lane 1, 394+393= lane 2, 396+398= lane 3). Only one combination of primers successfully amplified a set of genes (397+398 which produced cbei0712-0713) visible in lane 4. PCR products were run alongside a 1kb hyperladder (lane H). The band above was shown to be around 3,000 kb in length, as expected.

3.2.2 The cloning of PTS genes *cbei*0712-0713:

The amplified genes were then inserted into the plasmid pSC-A-amp/kan' (described in section 2.4) and transformed into SoloPack cells which were grown on LB agar containing ampicillin or kanamycin and X-gal. White and light blue colonies were patched onto a new plate (Figure 3.4) and screened for the insert using PCR as shown in Figure 3.5.

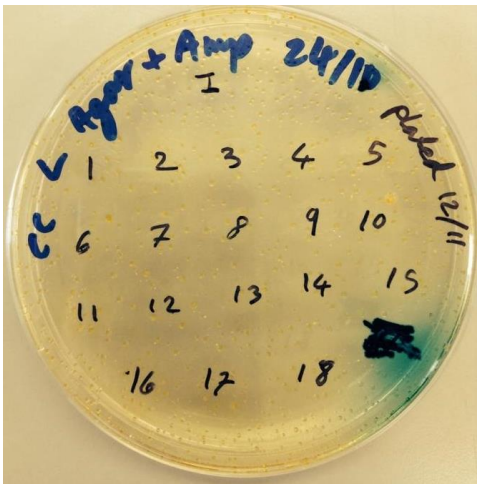


Figure 3.4. Colonies Patched onto LB Agar Plus X-Gal and Ampicillin.

The photo above shows colonies from the cloning stage after being patched onto LB agar with the addition of X-Gal plus Ampicillin. White/light blue colonies were presumed to contain the plasmid, carrying a cloned insert. One dark blue colony obtained from the transformation was used as a control.

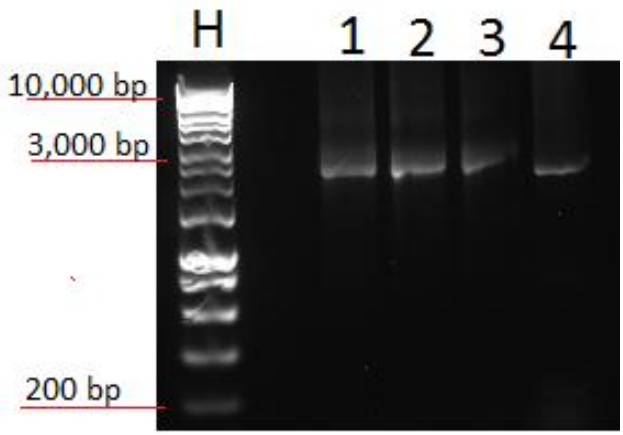


Figure 3.5. Screening for the *cbei*0712-0713 insert amplified by primers 397+398

Four colonies from the LB agar plate with ampicillin and X-Gal were that were screened by PCR after cloning were found to contain the insert (colonies 1, 2, 3 and 9). Lane 1= colony 1, lane 2 = colony 2, lane 3 = colony 3 and lane 4 = colony 9. Bands are shown next to a 1kb hyperladder (lane H). All four bands were shown to be roughly 3,000 bp in length.

Four colonies that contained plasmids with the correct insert were stored at a low temperature (4^0C) and restreaked onto a fresh plate when necessary. The plasmids from the four selected colonies (1, 2, 3 and 9) were purified using the Miniprep Kit (method in 2.7.1),

3.2.3 Determining the orientation of plasmid inserts *cbei0712-0713*:

The orientation of the insert from each plasmid obtained was determined using either the M13 Reverse primer or T7 promoter primer with the forward primer (397) or the reverse primer (398) in a PCR using the plasmid in question as template. Genes are under control of either by the *lac* or the T7 promoter in the StrataClone Vector (Appendix III) and so the orientation could be found by varying the primers used in the reaction. The M13 Reverse primer 372 and the T7 promoter primer 373 were paired with one of the primers from each initial reaction.

If the reverse primer from one of the initial reactions was paired with the M13 Reverse primer (M13R) and a product was formed through PCR, this would indicate that the insert was under the control of the *lac* promoter (the forward direction). However, if the forward primer from the initial reaction was paired with the M13 Reverse primer and a product was formed, this would suggest that the insert is not under the control of the *lac* promoter and so would be unlikely to be expressed.
Similarly, if the reverse primer from the initial reaction was used in a PCR with the T7 promoter primer and a product was formed, this indicates that the insert is under control of the T7 promoter (the reverse direction). If the forward primer produced a product when paired with the T7 promoter primer, it can be concluded that the insert is not under control of the *lac* promoter.

The orientation of all *cbei*0712-0713 inserts in plasmids 1, 2, 3 and 9 were investigated through several PCRs (Figure 3.6).

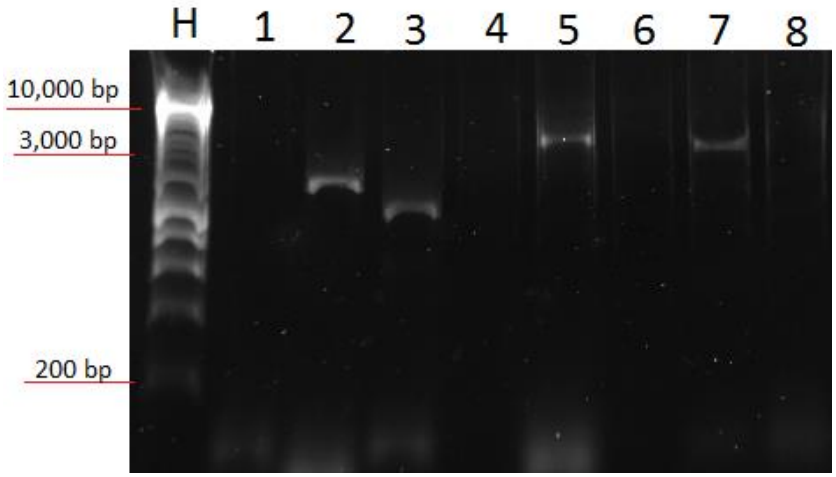


Figure 3.6. Screening for orientation of inserts cbei0712-0713 in vector

Screening for the insert of genes *cbei*0712-0713 in plasmid next to a 1kb hyperladder (H). Lane 1= Plasmid 1, M13 R + primer 398. Lane 2= Plasmid 1, M13 R + primer 397. Lane 3= Plasmid 2, M13 R + primer 398. Lane 4 = Plasmid 2, M13 R + primer 397. Lane 5= Plasmid 3, M13 R + primer + primer 398. Lane 6= Plasmid 3, M13 R + primer 397. Lane 7 = Plasmid 9, M13 R + primer 398. Lane 8 = Plasmid 9, M13 R + primer 397.

The results indicated that the insert of Plasmid 1 was in the reverse orientation whereas Plasmids 2, 3 and 9 had insert in the forward orientation. However, only plasmids 3 and 9 gave a PCR product of the expected size. The reason why the other plasmids gave unexpectedly small inserts is unknown, but it is possible that they were contaminated by a smaller product, which would have been able to ligate into the vector at a higher efficiency than a larger fragment.

3.2.4 The fermentation phenotype of *cbei*0712-0713:

Plasmid 3 was used in the transformation into *E. coli* ZSC113 cells. In order to view the mannose and glucose fermentation phenotypes of the transformed mutant, cells were patched onto MacConkey agar plates containing ampicillin and either mannose or glucose as the carbon source. The colour of the MacConkey agar after incubation determines the fermentation type of bacteria streaked on it. When colonies gain a pink/purple tinge and the red colour of the agar is retained, it shows that the bacteria can indeed ferment the carbon source added to the agar. However, when the colonies and surroundings are an orange or yellowish colour, it shows that the bacteria cannot ferment the added carbon source.

It appeared that the transformed mutants reacted positively to mannose, while reacting negatively on plates with glucose (Figure 3.7). However, there was very little growth on the plates containing mannose. The colonies were therefore restreaked onto plates with mannose but without ampicillin so that they could be compared with the untransformed mutant (Figure 3.8). Again, the transformed colonies appeared to give a positive result, but the small amount of growth made it difficult to determine whether or not they could ferment the sugar. There was substantial growth in the case of control colonies and they gave a negative fermentation result as anticipated. The transformation step was then repeated. The transformed cells on this occasion did grow well on MacConkey plates containing mannose, but showed a negative fermentation phenotype (Figure 3.9). While all plates showed a negative result, the MacConkey plates containing mannose retained a red tint, while plates with glucose had turned to an orange/yellow colour. The same results occurred when the colonies grown in Figure 3.9 were transferred onto MacConkey agar plates without antibiotic and grown alongside the untransformed *E. coli* ZSC113 mutant as the negative control (Figure 3.10).

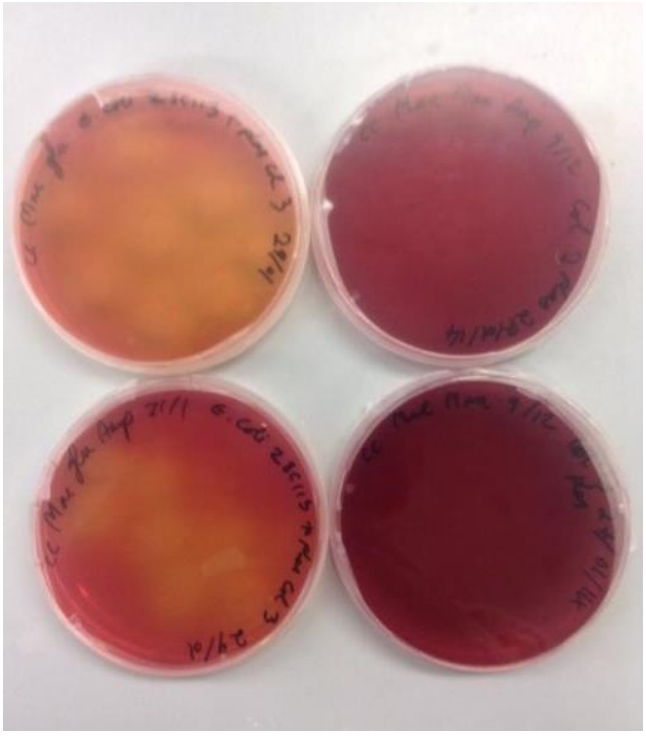


Figure 3.7. Colonies after transformation of *E. coli* ZSC113 with genes *cbei*0712-0713

Eight colonies that derived from the transformation were plated onto MacConkey agar containing ampicillin and either mannose (right) or glucose (left). Colonies showed a definite negative fermentation phenotype on glucose. The fermentation type appeared to

be positive on mannose, but there was little growth after the overnight incubation period and the results were therefore not conclusive.

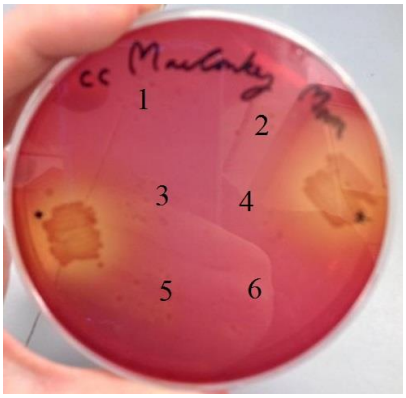


Figure 3.8. *E. coli* ZSC113 colonies transformed with plasmid containing *cbei* 0712-0713 next to the untransformed *E. coli* ZSC113 control

Transformed colonies (1-6) were restreaked in patches onto a new MacConkey mannose agar plate without ampicillin so that they could be compared to the untransformed mutant. Restreaked patches of colonies are marked by numbers placed above growth and the control marked by '*'. While the control grew well and gave a clear negative result, the transformed colonies barely grew and a definite result could not be obtained.

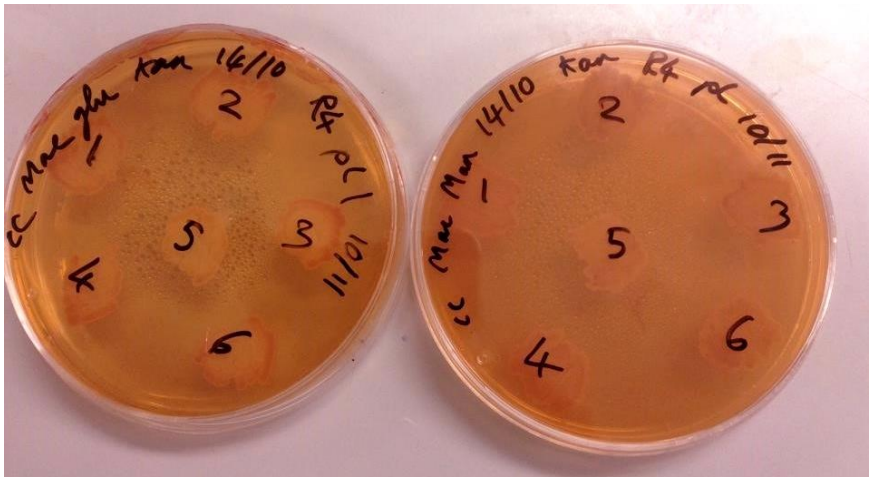
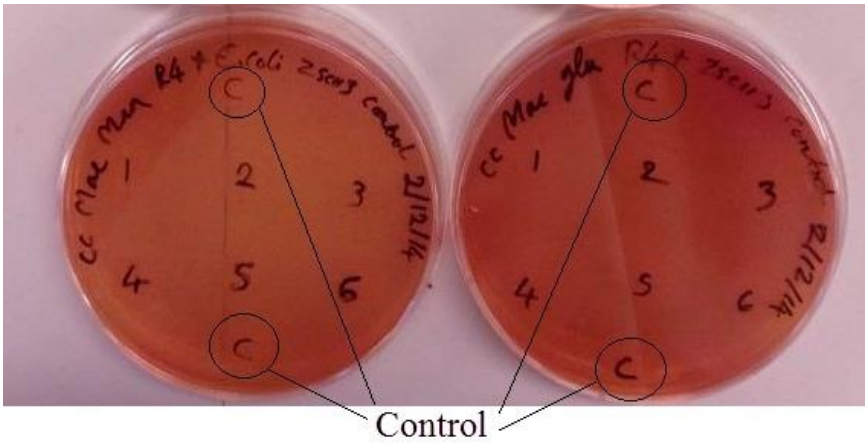


Figure 3.9. *E. coli* ZSC113 colonies transformed with plasmid containing genes *cbei*0712-0713

Colonies (1-6) from a second transformation with plasmid containing PTS genes *cbei*0712-0713 were spread onto MacConkey agar plates with kanamycin and either mannose (right) or glucose (left). All colonies showed a distinctly negative fermentation phenotype after incubation at 37^0C for 48 hours.



3.10. Transformed colonies with *cbei*0712-0713 genes with control

Transformed colonies (1-6) which initially grew on the MacConkey plates with kanamycin were later streaked onto fresh MacConkey plates without antibiotic. This was so that the colonies could be compared to the growth and fermentation of the untransformed mutant (the control marked 'C'). All colonies showed a negative fermentation phenotype on the plates with mannose (left) and glucose (right) as the additional carbon source after 48 hours of incubation.

3.2.5 The Utilisation of Mannose and Glucose by Cells Transformed with *cbei* 0712-0713:

Due to the inconsistencies seen in the fermentation analysis, it was decided to follow the uptake of mannose and glucose by cells in culture (section 2.10) to examine whether or not mannose could be taken up by the transformed cells. A selected *E. coli* ZSC113 transformant was were grown overnight in LB medium containing kanamycin before being inoculated into fresh LB medium with kanamycin and either mannose or glucose. As free monosaccharides, particularly glucose, are usually favoured by bacteria over other energy sources, it would be expected that cells would utilize either sugar if they had the means to do so. However, alternative carbon sources in the LB medium would allow cells to grow, even if neither mannose nor glucose could be used by the cells. The optical density (OD_{600}) of cultures was measured hourly to follow growth, and sugar utilisation was followed by measuring the concentration in the culture supernatant (Figure 3.11).

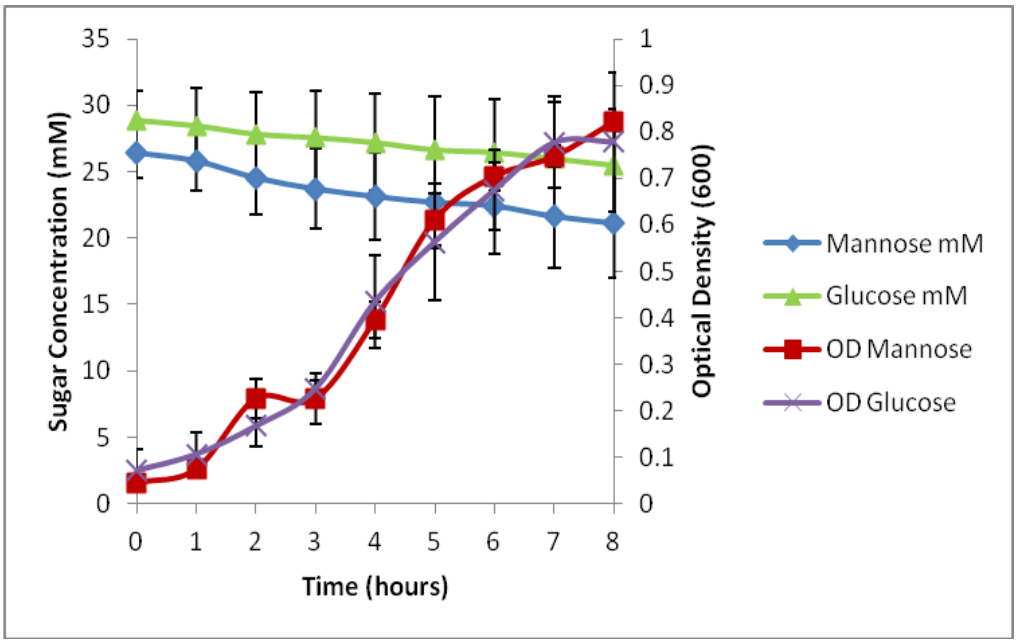


Figure 3.11. Mannose and glucose utilisation by cells transformed with *cbei*0712-0713

Escherichia coli mutant ZSC113 was transformed with plasmid containing PTS-encoding genes *cbei*0712-0713 from *Clostridium beijerinckii* and grown in LB medium containing 25mM of either mannose or glucose. The sugar concentration and OD of each sample was measured every hour. Triplicate cultures were grown and error bars show the standard deviation.

Essalem (2014) completed sugar utilization experiments with untransformed *E.* coli ZSC113 on mannose and glucose to be used as negative controls., using the same OD, sugar concentrations and time period shown in Figure 3.11. His results showed a very slight decrease in glucose over a period of eight hours and a barely visible decrease in mannose over the same time period.

It was shown that although cells did grow in media both with and without added sugar, there was little change in either mannose or glucose over the course of the culture. Although the mannose concentration did decrease by around 15%, this is a much smaller change than seen for E. coli ZSC113 transformed with cbei0751 gene which encodes a glucose-mannose PTS (around 50%). However, this change was also much larger than Essalem's untransformed control. From this, it can be concluded that the insertion of genes *cbei* 0712-0713 did not give the mutant the ability to utilise glucose or mannose to a significant extent.

3.3. Phosphotransferase system Cbei3871-3874:

In the next phase of the project, primers were obtained in order to amplify the genes of another system; *cbei*3871-3874 (Figure 3.12)

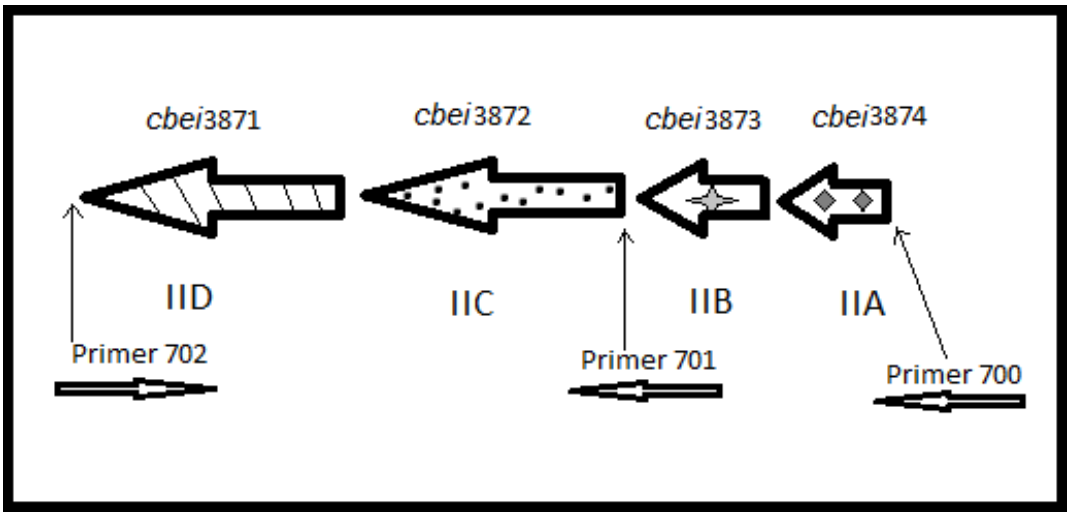


Figure 3.12. The genomic organisation of *Clostridium beijerinckii* NCIMB 8052 genes *cbei*3871 to *cbei*3874.
The large arrows represent the genes for a phosphotransferase system of *Clostridium beijerinckii* and their directions while the text above shows which gene each arrow represents. The labels IID, IIC, IIB and IIA below show which part of the system they encode. Numbers below the arrows represent the primers used as described in table 2.1 and their binding locations within the genome. The smaller arrows below show whether a primer works in the forward or reverse direction. The precise primer annealing positions are shown in Appendix I.

3.3.1 The amplification of PTS genes *cbei*3871-3874:

Two pairs of primers (700+702 and 701+702) were used in a PCR to amplify the genes shown in Figure 3.12. On the first attempt, primers 700+702 produced a product of the correct length (3,000 bp) as seen in Figure 3.13A, whereas 701+702 failed to produce any product. The PCR was carried out again using primers 701+702, this time lowering the annealing temperature from 56^0C to 48^0C. This had the desired effect and the PCR produced one clear band at around 1,500bp (Figure 3.15 B). In order to separate the desired band from the other smaller bands produced by 700+702, more PCRs using higher annealing temperatures were initiated. It was found that increasing the annealing

temperature of the PCR from 53^{0}C to 56^{0}C was effective and lead to the production of one strong band around the size of 3,000bp.

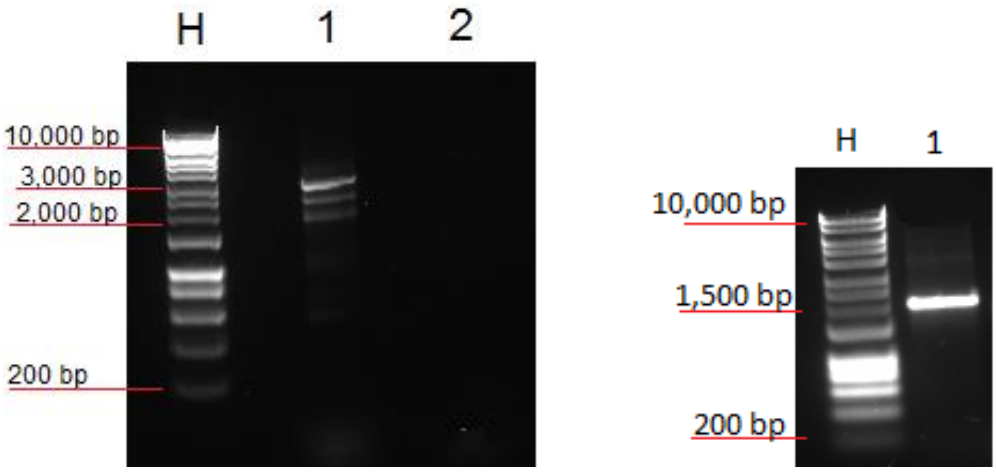


Figure 3.13. The amplification of genes *cbei*3871-3874 by PCR
The bands are shown next to a 1 kb hyperladder. A; lane 1= *cbei*3871-3874 amplified with primers 700 and 702. lane 2= *cbei*3871-3872 amplified with primers 701 and 702. B; lane 1= second attempt at amplifying *cbei*3871-3872. H= hyperladder

3.3.2 Screening for insert of *cbei*3871-3874 PTS in plasmid:

On the basis that it would be better to clone the entire PTS for functional analysis, the PCR products obtained with primers 700+702, containing genes for all three domains of the respective PTS, were then cloned in the StrataClone plasmid. Colonies obtained from the transformation were plated onto LB medium with ampicillin and X-Gal. The screening of white colonies showed that several from each set contained an insert. As there were many colonies to screen, they were initially screened in sets of three. As shown in Figure 3.14, colonies 1-3 were all found to contain the insert amplified with primers 700+702 when screened individually.

Plasmids were isolated from colonies 1 and 2 using the MiniPrep Kit. Once it was determined that plasmid was present in both samples, another PCR was set up to screen the samples for insert to ensure its presence before moving on to the next stage of the experiment.

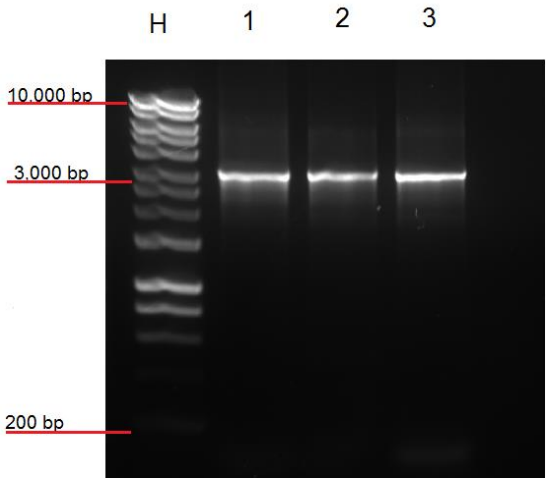


Figure 3.14 Screening for insert in 3 selected colonies

After the cloning of genes amplified by primers 700+702, three resulting colonies were screened for the insert. All colonies produced a clear band of the expected length next to the 1 kb hyperladder, showing that they did indeed contain the insert.

3.3.3. Screening the orientation of the insert of *cbei*3871-3874 PTS plasmids:

Once it had been established that the plasmid did indeed contain the correct insert, the orientation of the insert was found using the same method of PCRs described in Sections 2.6. and 3.2.3 Two PCRs were completed for each of the two plasmids ; one using the reverse cloning primer and M13 Reverse primer (702+372) and another using the forward cloning primer with M13 Reverse primer (700+372) as shown in Figure 3.15. Both plasmids were shown to contain insert in the forward direction.

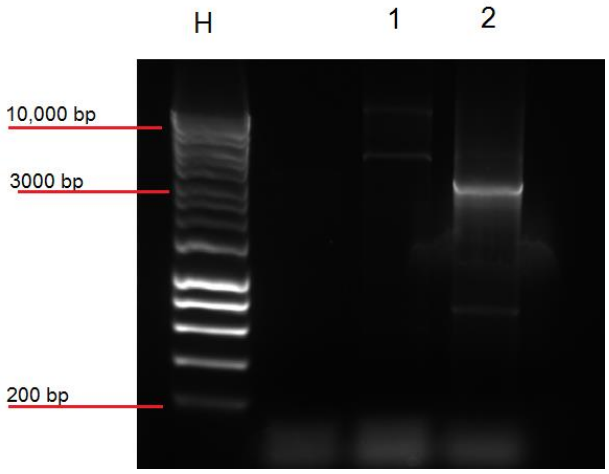


Figure 3.15: Screening for orientation of plasmid insert *cbei*3872-3874

To determine whether the insert was positioned in the forward or reverse direction within the plasmid, an orientation PCR was carried out. Lane 'H' contains the hyperladder, '1' contains the PCR product produced using primers 372+700 and '2' contains the product produced using primers 372+702. Results indicate that insert is in the forward orientation.

As a PCR product was produced using M13 Reverse primer with the reverse cloning primer (372+702), this indicated that the insert is in the forward orientation within the plasmid, meaning the genes are likely to be expressed when transformed into the *E. coli* ZSC113 mutant.

3.3.4 Fermentation phenotype of PTS *cbei*3871-3874 in transformed mutant:

The plasmids were then transformed into the mutant *E. coli* ZSC113 so that the fermentation phenotype could be observed. Colonies that grew following the transformation were streaked onto LB agar with either ampicillin or kanamycin and left to grow for 24 hours before being streaked onto MacConkey agar , also containing either ampicillin or kanamycin, and left to incubate for another 48 hours. Both transformants showed a negative fermentation type on both mannose and glucose (Figure 3.16).

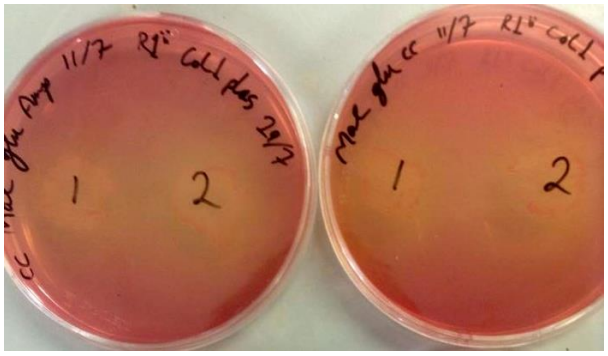


Figure 3.16. *E. coli* ZSC113 colonies transformed with PTS genes *cbei*3871-3874

Following the transformation of plasmid into *Escherichia coli* ZSC113, colonies that grew on the initial LB agar with ampicillin were streaked onto MacConkey agar containing ampicillin and either mannose (left) or glucose (right) as a carbon source. Both colonies showed a negative fermentation phenotype for both sugars.

Since only two colonies were gathered in the first transformation, another was completed using the same plasmid so that a greater sample size would be obtained. While the MacConkey plate containing glucose appeared slightly paler in colour than the mannose plate, all colonies gave a negative result. After the colonies had been on MacConkey agar containing ampicillin for at least 24 hours, they were restreaked onto fresh plates without antibiotics so that the transformants could be compared to the untransformed control colonies (Figure 3.17). Again, there was no evidence for glucose or mannose fermentaion by the transformants.

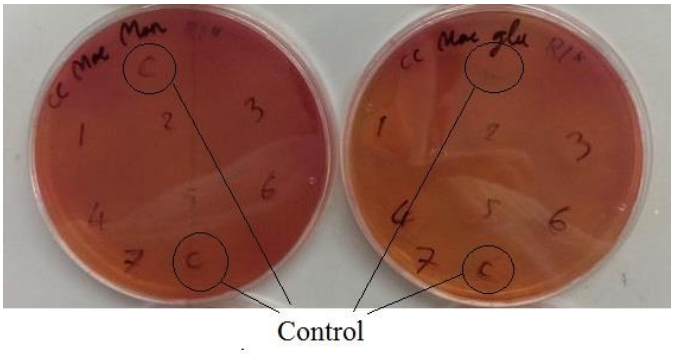


Figure 3.17. Transformed colonies containing *cbei*3871-3874 genes and the *E. coli* ZSC113 control.
Transformed colonies which initially grew on the MacConkey plates with ampicillin were later streaked onto fresh MacConkey plates without antibiotic. This was so that the colonies could be compared to the growth and fermentation of the untransformed mutant (the control marked 'C'). All colonies showed a negative fermentation phenotype on the plates with mannose (left) and glucose (right) as the additional carbon source.

3.3.5 Utilisation of mannose and glucose and growth of cells transformed with *cbei*3871-3874 and *cbei*0958:

As with the cells that had been transformed with plasmid containing *cbei*0712-0713, the transformed cells containing the genes *cbei*3871-3874 were tested for utilisation of mannose and glucose in LB medium.

The transformed cells were compared for absorption of mannose and glucose and for overall cell growth in the culture (Figure 3.18). The levels of both mannose and glucose decreased very slightly, but not to a level that would suggest that the inserted PTS genes had any role in utilisation of the sugars. Both results for sugar concentration highly resembled Essalem's (2014) negative controls for mannose and glucose. The OD rose as expected over the eight hour period, suggesting that the cells were relying on the nutrients available in the LB medium rather than the added sugars.

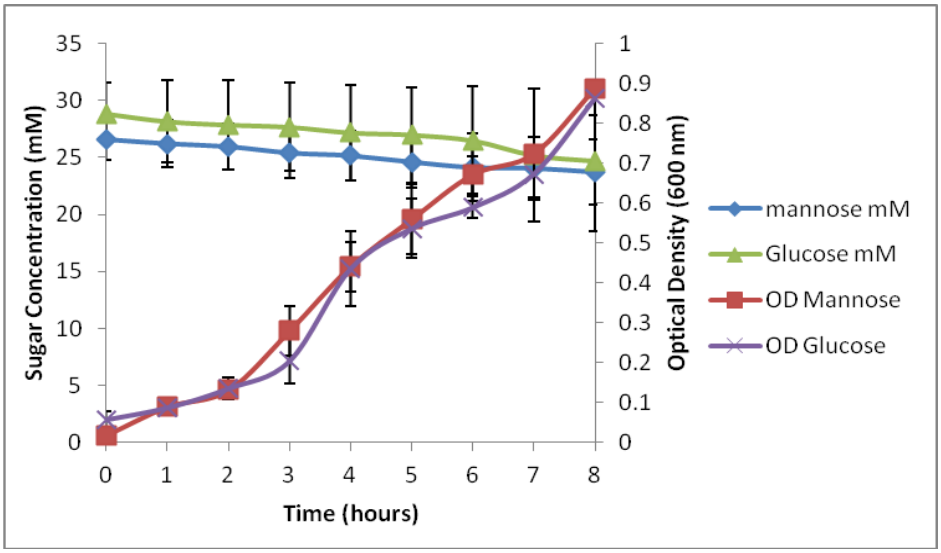


Figure 3.18. Mannose and glucose utilisation by cells transformed with *cbei*3871-3874

Escherichia coli mutant ZSC113 was transformed with PTS encoding genes from *Clostridium beijerinckii* and grown in LB media containing 25mM of either mannose or glucose. The sugar concentration of each sample was measured every hour over a set period of time.

3.4. Phosphotransferase system *cbei*0955-0958:

The third phosphotransferase system from *C. beijerinckii* to be transformed into the *E. coli* ZSC113 mutant was *cbei*0955-0958 (Figure 3.19).

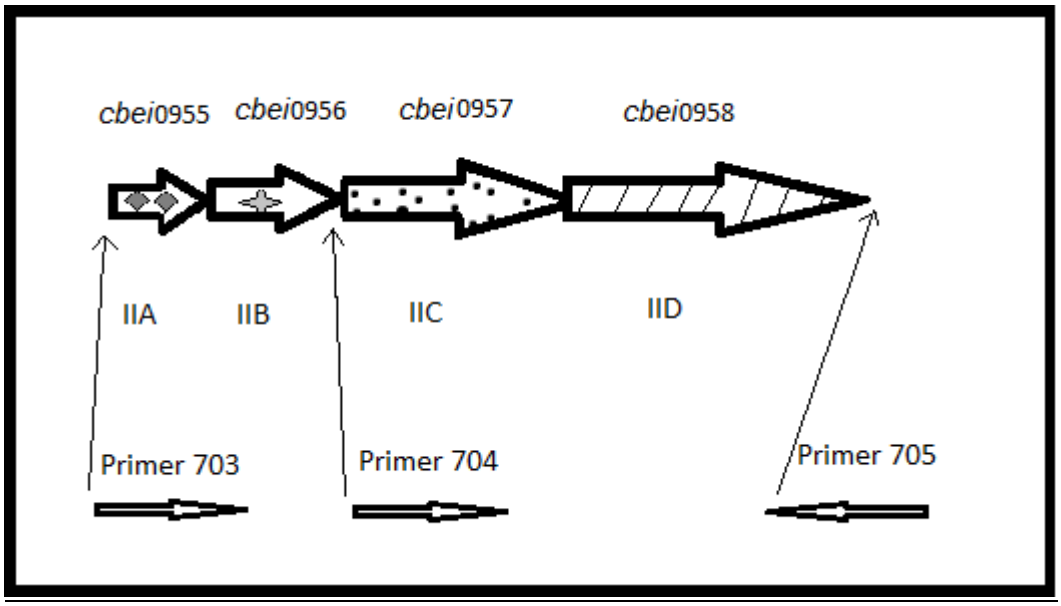


Figure 3.19. The genomic organisation of *Clostridium beijerinckii* NCIMB 8052 genes *cbei*0955 to *cbei*0958
The large arrows represent the genes for a phosphotransferase system of *Clostridium beijerinckii* and their directions while the text above shows which gene each arrow represents. The labels IID, IIC, IIB and IIA below show which part of the system they encode. Numbers below the arrows represent the primers used as described in table 2.1 and their binding locations within the genome. The smaller arrows below show whether a primer works in the forward or reverse direction. The precise primer annealing positions are shown in Appendix I.

3.4.1 The amplification of phosphotransferase system *cbei*0957-0958:

Primers 703+705 (all domains) and 704+705 (IIC and IID domains) were used in a PCR to amplify the genes shown in Figure 3.19. The first attempt with primers 703+705 produced products of the correct length (around 2,500bp) as seen in Figure 3.20 A., while the primers 704+705 produced three very faint bands. The PCR was carried out again using primers 704+705, with a lower annealing temperature of 48^0C instead of 60^0C. This time, one clear band was produced of the correct size (around 1,500bp) shown in Figure 3.20 B.

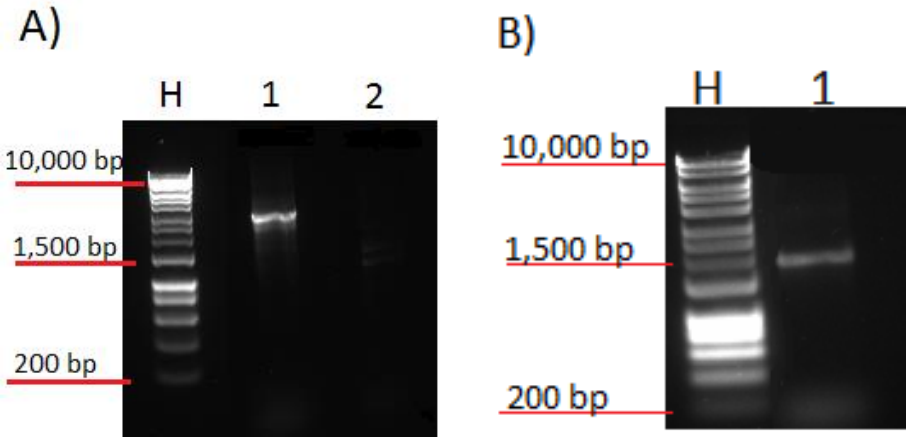


Figure 3.20. Amplification of *cbei*0955-0958

A. The image shows the PCR product from primers 703+705 (lane 1) containing *cbei*0955-0958 and primers 704+705 (lane 2) with *cbei*0957-0958 next to a 1kb hyperladder (H).
B. The second PCR using primers 704+705 with a lower annealing temperature of 48^0C instead of 60^0C.

3.4.2 Screening for insert of *cbei*0957-0958 PTS in plasmid:

The PCR products were cloned into the StrataClone plasmid. Colonies produced from the transformations were plated onto LB medium with ampicillin and X-Gal, and white colonies were screened for the presence of a cloned insert. No colonies that were transformed with the plasmid containing *cbei*0955-0958 were shown to possess an insert. On the other hand, several clones transformed with *cbei*0957-0958 did contain an insert. As there were many colonies to screen, they were initially screened in sets of three.

When colonies 1-3 were screened, it was clear that no insert was present. A band appeared in the PCRs made from colonies 4-6 and 7-9, so colonies 4-9 were then screened individually (Figure 3.21). Colonies 4 and 7 produced strong bands of the correct length (around 1,500bp). These two colonies were then incubated overnight in LB at 37^0C to be used for a plasmid prep. Plasmid was successfully obtained from both colonies.

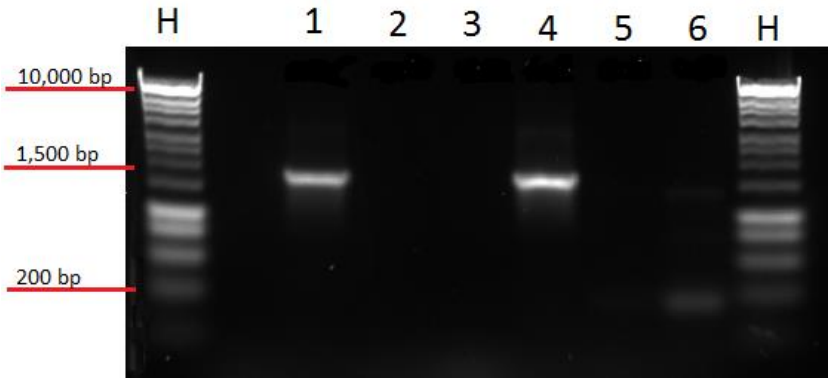


Figure 3.21. Screening of colonies 4-9 after cloning of *cbei*0957-0958

Colonies 4-9 were screened for the presence of insert. Lane H=hyperladder, lane 1= colony 4, lane 2 = colony 5, lane 3= colony 6, lane 4 = colony 7, lane 5= colony 8 and lane 6= colony 9.

3.4.3. Screening the orientation of the insert *cbei*0957-0958 PTS in plasmid:

The orientation of the insert was found using the same method of PCRs mentioned in Section 2.7. Two PCRs were completed ; one using the reverse cloning primer and M13 Reverse primer (705+372) and another using the forward cloning primer with M13 Reverse primer (704+372) as shown in Figure 3.19. The PCR using primers 705+372 produced the strongest product and so the insert was assumed to be in the forward orientation (Figure 3.22).

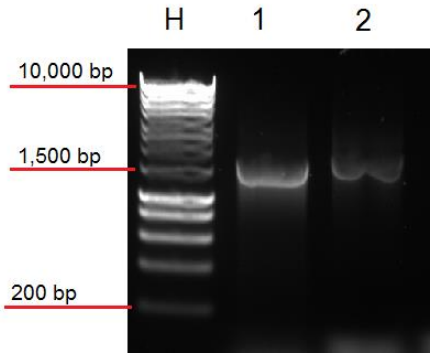


Figure 3.22. Orientation screening of inserted *cbei*0957-0958 genes
Lane 'H'= hyperladder. Lane 1= M13 Reverse + reverse cloning primer 705. Lane 2 = M13 Reverse + forward cloning primer 704. The insert is believed to be in the forward orientation.

3.4.4 Fermentation Phenotype of PTS Cbei3871-3874 in Transformed Mutant:

Plasmids containing the insert amplified with primers 704+705 were prepared from two colonies using the MiniPrep Kit. These plasmids were then transformed into the mutant *E. coli* ZSC113 so that the fermentation phenotype could be observed. Colonies that grew following the transformation were streaked onto LB agar with amp or kan and left to grow for 24 hours before being streaked onto MacConkey agar containing either mannose or glucose and left to incubate for another 48 hours.

While the plate containing mannose retained more red colouring than the plate with glucose, a negative fermentation phenotype was seen on both plates (Figure 3.23).

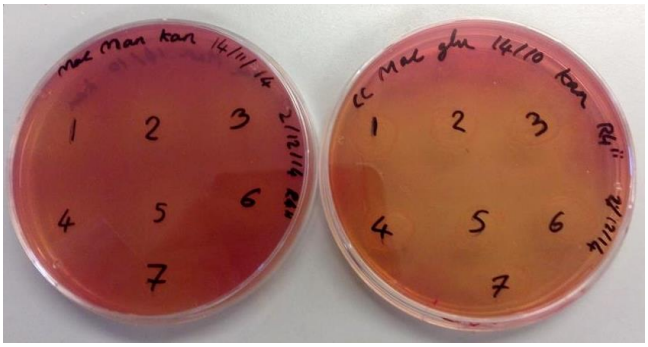


Figure 3.23. Transformed colonies of *E. coli* ZSC113 with genes *cbei*0957-0958

Colonies that were successfully transformed with the plasmid containing PTS encoding genes from *Clostridium beijerinckii* were streaked onto MacConkey agar plates so that their fermentation phenotypes could be observed. One plate (left) contained mannose while the other (right) contained glucose as the added carbon source. The agar contained kanamycin so that the colonies would retain the plasmids during growth.

The colonies were then picked from the MacConkey plates containing kanamycin and restreaked on to new plates without the addition of any antibiotic so that they could be compared to the untransformed mutant (Figure 3.24). Again, while the plate containing mannose retained most of its colour, all colonies showed a negative fermentation phenotype. Shades of orange could be seen around all the colonies streaked onto the plate

containing mannose, showing that the colonies cannot use the mannose within the medium. Glucose was also not fermented.

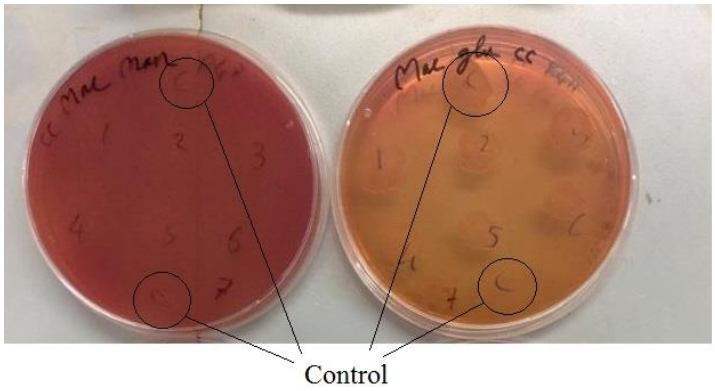


Figure 3.24. Transformed colonies of *E. coli* ZSC113 with genes *cbei*0957-0958 plus untransformed mutant
Colonies that had been successfully transformed with the plasmid and had grown on MacConkey agar with kanamycin were restreaked onto fresh MacConkey plates without an antibiotic. This was done so that the untransformed *Escherichia coli* ZSC113 (the control marked 'C'), which is not resistant to kanamycin, could grow with transformed colonies for comparison. Colonies were spread onto one plate containing mannose (left) or glucose (right) as a carbon source. Both isolates showed a negative fermentation phenotype for both sugars.

3.4.5 Utilisation of mannose and glucose and growth of cells transformed with *cbei*0957-0958:

Colonies of *E. coli* ZSC113 which were transformed with genes *cbei*0957-0958 were selected and grown in LB containing either mannose ir glucose to see if either sugar would be utilised (Figure 3.25).

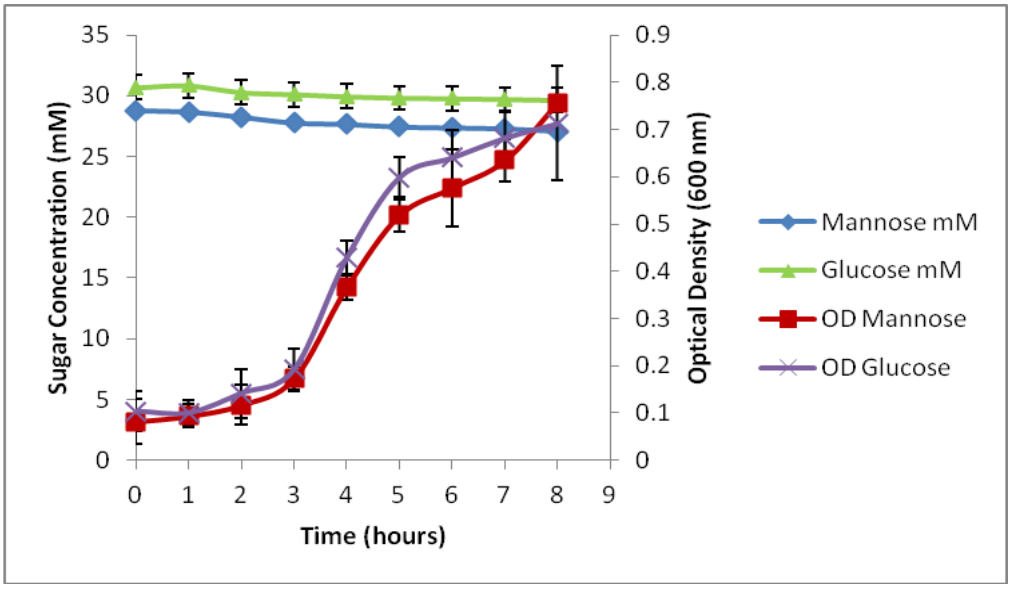


Figure 3.25. Mannose and glucose utilisation by cells transformed with *cbei*0957-0958

Escherichia coli mutant ZSC113 were transformed with plasmid containing genes *cbei*0957-cbei0958. Results shown are the averages of triplicates and error bars represent the standard deviation of each set of three results. Error bars for sugar concentration are hidden by the markers.

The results from transformed cells grown with mannose and glucose were similar and also highly resembled the negative controls from Essalem's (2014) results. There was no significant decline in mannose or glucose, but a steady growth in cells was observed. This indicates that neither mannose nor glucose was being used for cell growth.

3.5 The Phosphotransferase System Cbei0963-0966:

The final system examined was the one encoded by genes *cbei*0963 to *cbei*0966. Primers were designed for the amplification of these PTS genes. One pair amplified all four genes (*cbei*0963-0966) and the other pair amplified only the IIC and IID domains (*cbei*0965-0966). A diagram of the genes and the primers is shown in Figure 3.26.

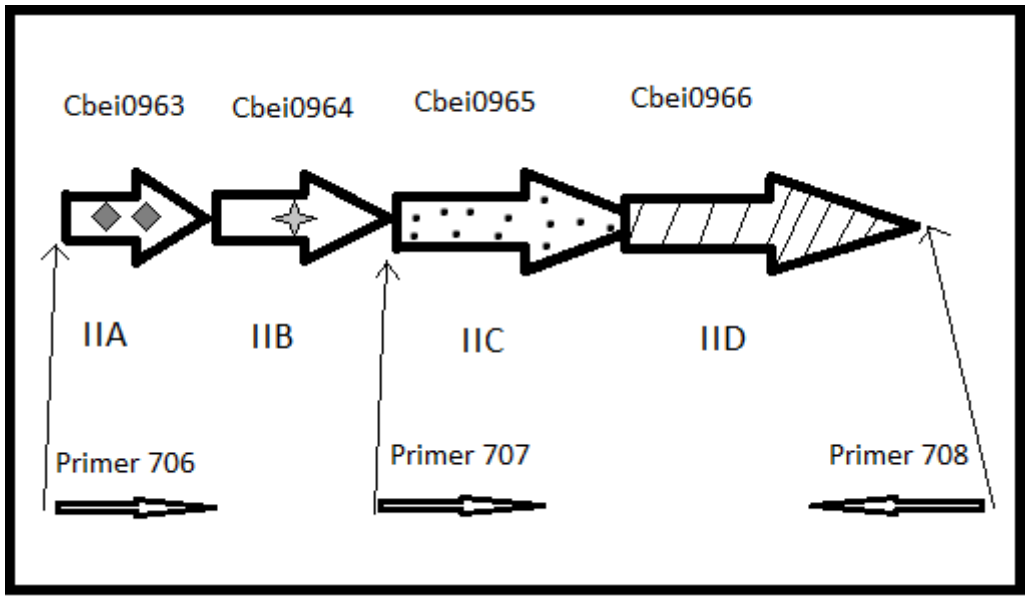


Figure 3.26. The genomic organisation of *Clostridium beijerinckii* NCIMB 8052 genes 0963 to 0966
Large arrows represent the order and direction of genes *cbei*0963-0966 within the genome of *Clostridium beijerincki*i. The label above each of the large arrows shows the name of each gene respectively. The labels IIA, IIB, IIC and IID below each large arrow show which domain of PTS enzyme II each gene encodes. Small arrows below each of the large arrows represent the primers used to amplify these genes and whether they are in the forward or reverse direction. The precise primer annealing positions are shown in Appendix I.

3.5.1 The amplification of genes *cbei*0963-0966:

PCR was initially set up with an annealing temperature of 56^0C, but no product was produced. The annealing temperature was then gradually lowered until eventually product was produced. Bands were formed when the annealing temperature was at 37^0C, 36^0C and 35^0C, but not any higher or lower. Primers 707+708 produced a strong single band of the expected size of around 1,500bp, whereas primers 706+708 produced several bands of low

intensity, the largest being of 4,000bp on size. An attempt was made to reduce the number of bands by repeating the PCR at higher annealing temperatures, but this was unsuccessful. It was therefore decided to use only the amplified product obtained with primers 707 and 708 for the next stage.

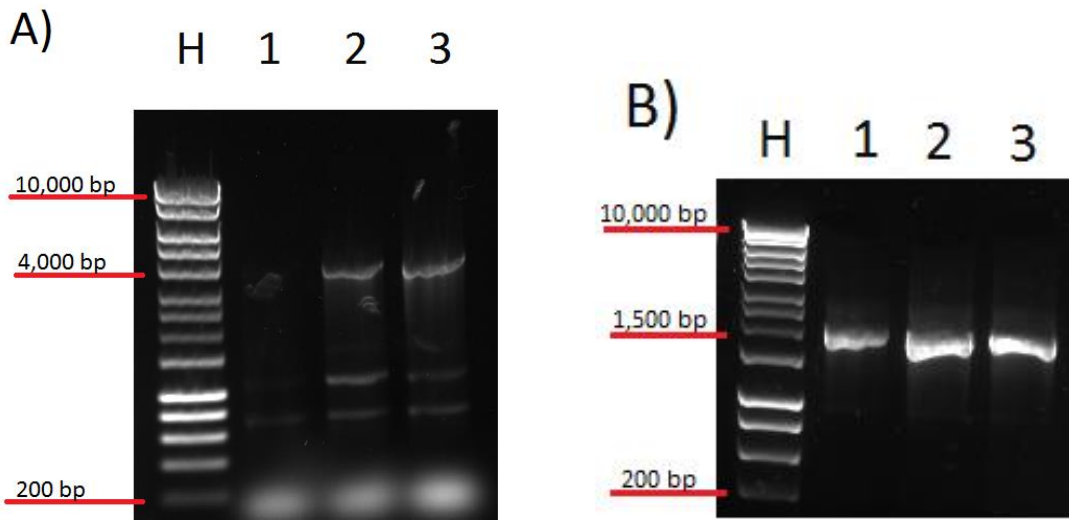


Figure 3.27. The amplification of A: genes *cbei*0963-0966 and B: genes *cbei*0965-0966

Annealing temperatures used were 35, 36 and 37^{0}C as no bands were produced when higher temperatures were used. Two sets of primers were used to amplify the phosphotransferase system. Column H marks the hyperladder which was 1kb in both A and B.

A. Shows the amplification of genes *cbei*0963-0966 using primers 706+708 using annealing temperatures 35^{0}C (lane 1), 36^{0}C (lane 2 and 37^{0}C (lane 3). Bands were visible in all three lanes at 4,000bp as well as two smaller bands in each lane.
B. Shows the amplification of genes *cbei*0965-0966 using primers 707+708 using annealing temperatures of 35^{0}C (lane 1), 36^{0}C (lane 2) and 37^{0}C (lane 3). Bands at around 1,500bp were shown to be present in all three lanes.

3.5.2 The cloning of PTS genes *cbei0965-0966:*

The amplified DNA was ligated into plasmid pSC-A-amp/kan and transformed into SoloPack cells. Colonies that were white or light blue in colour that grew on the LB agar containing ampicillin or kanamycin and X-Gal were screened as described in section 2.5.

The PCR screening of several colonies showed that one contained inserted DNA whereas a few others showed only very faint bands (Figure 3.28).

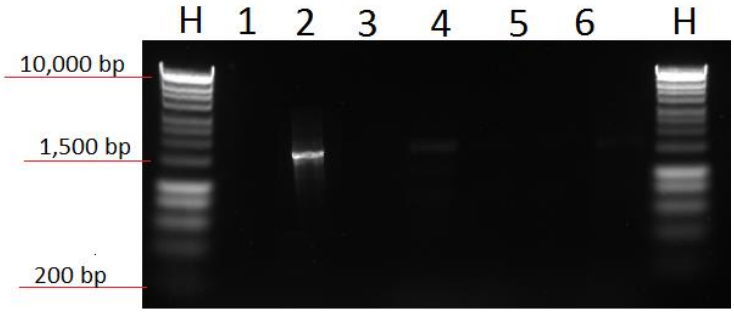


Figure 3.28. Screening for genes *cbei*0965-0966

Several colonies produced from the cloning stage were screened for the insert by PCR. The insert contains the genes (*cbei*0965-0966). The bands are shown next to a 1kb hyperladder (H). One colony produced a strong band of the expected length (lane 2) and another shows a fainter band of the same length (lane 4). No other colonies were shown to contain insert.

3.5.3 Screening for the orientation of genes *cbei*0965-0966:

The plasmid from Colony 2 was purified using the miniprep kit and then screened to determine the orientation of the insert. A PCR using the M13 Reverse primer with the reverse cloning primer (372+708) produced a strong band whereas PCR using the M13 Reverse primer and the forward cloning primer (372+707) produced a fainter band. From this it was deduced that the insert in Colony 2 was in the forward orientation (Figure 3.29).

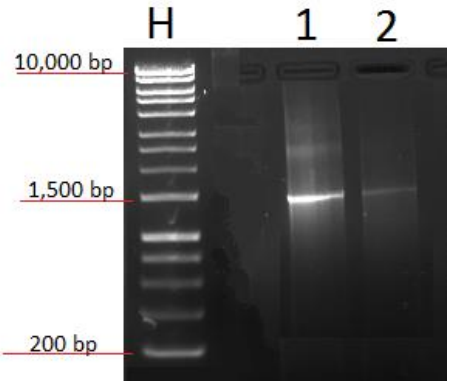


Figure 3.29. Screening for the orientation of genes *cbei*0965-0966

The purified plasmid from Colony 2 was screened for the orientation of its insert. H= hyperladder. Lane 1 = M13 Reverse primer + reverse primer (372+ 708). Lane 2 = M13 Reverse primer + forward primer (372+707). The strongest band was seen in lane 1 and so the insert is believed to be in the forward direction.

3.5.4 The fermentation phenotype of *cbei*0965-0966 in transformed mutant:

The purified plasmid from both Colony 2 and Colony 4 were transformed into the *E. coli* mutant ZSC113 and grown on LB agar plates containing amp or kan. Colonies that grew were then patched onto MacConkey containing amp or kan, and either mannose or glucose so that the fermentation phenotype of the cells could be observed. For this system, the results showed a positive fermentation of mannose and a negative fermentation of glucose (Figure 3.30).

The transformed cells were then compared to the fermentation phenotype of the mutant by restreaking the colonies onto MacConkey agar without antibiotic and with either mannose or glucose as the source of carbon. Again, the transformed mutant was shown to ferment mannose, but not glucose while the untransformed mutant could not ferment either sugar (Figure 3.31).

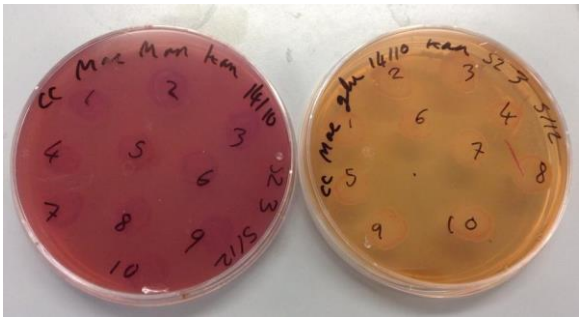


Figure 3.30. Colonies Spread onto MacConkeyAgar Plates Containing Kanamycin and Mannose or Glucose
Colonies that grew after the transformation stage were spread onto fresh MacConkey plates containing kanamycin and either mannose (left plate) or glucose (right plate) as the carbon source. The plate containing mannose shows a strong positive fermentation reaction, whereas the plate containing glucose shows a negative result.

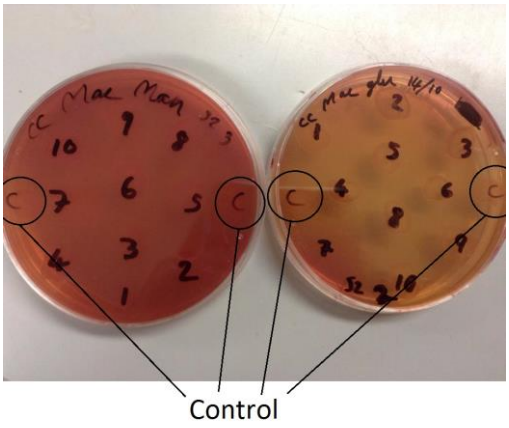


Figure 3.31. Colonies Spread onto MacConkey Agar Plates Containing Mannose or Glucose.with the Control (*E.coli* ZSC113).
Colonies from the plates in figure 3.30 were restreaked onto fresh MacConkey agar plates without kanamycin so that the untransformed *E.coli* ZSC113 could grow alongside them as the control. As with the plates shown in figure 3.30, the colonies showed a positive result for fermentation on the plate containing mannose (left) with the exception of the control colonies which were a pale orange (just seen in image). The colonies on the plate containing glucose all showed a negative fermentation result, including the control.

3.6 Sequencing of *E.coli* mutant ZCS113 PTS Genes:

The mutant strain *Escherichia coli* ZSC113 was isolated in 1975 following chemical mutagenesis of the wild type (Curtis and Epstein, 1975). While it is known for its inability to transport and phosphorylate glucose or mannose, the exact location of the mutations within its genome has remained unknown. It was decided that locating these mutations would be beneficial as it could be used to determine whether any components of Enzyme II remained functional, even if the full system is not. If the IIA and IIB domains were found to be altered, this may have been the reason why some of the cloned phosphotransferase systems which only included the IIC and IID domains could not complement the mutant for transport and phosphorylation of mannose.

3.6.1 Amplification of *E. coli* ZSC113 Mannose PTS

The *E. coli* mannose PTS is encoded by the *manXYZ* genes. *manX* encodes the IIA and IIB domains, while *manY* and *manZ* encode the IIC and IID domains respectively. Primers were designed so that all domains of Enzyme II encoded by the *manXYZ* genes could be amplified. Several attempts of the amplification were required and the annealing temperature was eventually lowered to 35-37^{0}C (Figure 3.32). Once the genes had been successfully amplified, they were cloned into plasmid Cloning Vector pSC-A-amp/kan so that the genes could be sequenced.

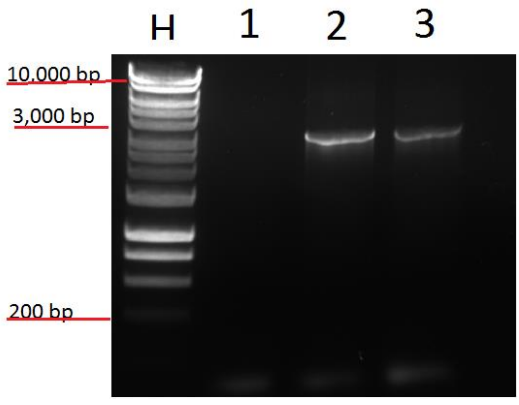


Figure 3.32. Amplification of the *E. coli manXYZ* genes by PCR.

The above figure shows the result after amplification using different annealing temperatures next to a 1 kb hyperladder (H). The amplified products are around 3,000kb in length. Lane 1: Empty. Lane 2: PCR with annealing temperature set to 35^{0}C. Lane 3: PCR with annealing temperature set to 37^{0}C.

3.6.2 Screening for *E. coli* Man PTS insert

As with the PTSs of *C. beijerinckii*, the amplified *E. coli* PTS was ligated into plasmid pSC-A-amp/kan and cloned in SoloPack cells. Two of the colonies screened following cloning were found to possess the required insert (Figure 3.33). Orientation of the insert was not important in this case, and so was not determined.

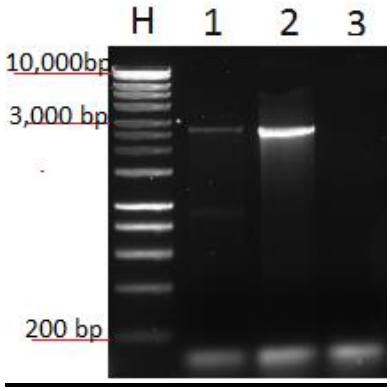


Figure 3.33. Screening of clones of the *E. coli manXYZ* genes in *E. coli* SoloPack cells.

Following the cloning, colonies were screened for the insert (lanes 1-3) next to a 1kb hyperladder (H). Lane 1 shows a faint band, whereas the colony screened in lane 2 produced a strong band. The colony screened in lane 3 did not possess the insert.

3.6.3: Sequencing Mannose PTS of E.coli ZSC113

A large plasmid prep was prepared using the Midi kit in order to collect a large amount of plasmid to be used for sequencing. The entire sequence of cloned genes was determined on both strands. The gene sequence of the mutated *manXYZ* PTS was then compared base by base to the wild type sequence in order to detect exactly where any mutations occurred (Appendix II). The sequence of 3,000 bp was found to contain a total of 5 mutations. The next step was to determine what changes these mutations caused in the encoded proteins. To do this, the DNA sequences were translated into amino acid sequences.

The *manX* gene encoding the II AB domains had only one mutation; a codon which initially encoded glutamine (CAG) became a stop codon (TAG) when the C was replaced with a T at codon 51. This would mean that translation of *manX* was disrupted by a nonsense mutation and that the AB domains of the mannose PTS were unlikely to be functional.

The first mutation found in the *manY* gene was the replacement of a T base (GGT) with a C base (GGT) at codon 40. Both codons encode cysteine and so this mutation should not have had any effect on the final protein. The second was also substitution of a T base (AAT) by a C base (AAC) at codon 58. Both are codons encoding asparagine. The third mutation replaced an A base with G in codon 153, turning the codon ATT into GTT. This results in a codon encoding valine instead of isoleucine. Both amino acids are non-polar with a neutral pH, so may not have altered the final protein drastically.

The mutation found in the *man*Z gene changed codon 20 which encoded the non-polar amino acid glycine (GGC) before an A base substituted the second G base (GAC), causing the codon to encode the polar amino acid aspartic acid. The effect of this change on the final protein is currently unknown.

4. Discussion:

The use of biofuels has recently been attracting more attention due to the environmental benefits in comparison to the use of fossil fuels. Biofuels are a renewable source of energy, several of which are carbon neutral. They are quickly created through fermentation as opposed to their fossil fuel counterparts which can take up to 650 million years or more to form (Mann et al, 1999). Butanol was widely produced in the mid-twentieth century until its production could no longer compete economically with the extraction of fossil fuels. As fossil fuels are rapidly being consumed, they are unsustainable given the amount of time required to create them. Because of this, interest towards biobutanol and other biofuels has increased in recent years as an alternative fuel source will be needed in the near future.

Butanol is produced by fermentation of starches, mono and disaccharides with the use of solventogenic bacteria. As it would be more acceptable not to use plant materials that could be used in food production, researchers are becoming interested in using plant based waste products for biofuel production. A great number of sugars are found in plant waste and so in order to make the fermentation process as efficient as possible, it is vital to understand how all aspects of the process work, including sugar selection and absorption.

The main method of sugar absorption in anaerobic, fermentative bacteria is the phosphotransferase system (PTS). This system usually consists of five domains carried on at least three separate proteins. The proteins Enzyme I, phosphocarrier HPr and an Enzyme II complex are found within all PTSs.

Carbon catabolite repression (CCR) is the term given for bacteria preferring one type of sugar over another. This means that while a preferred sugar is present (most commonly glucose), the absorption of other sugars present is repressed until the favoured source is depleted. This is not ideal in biofuel production as preferably all sugars would be fermented in the shortest time possible. In order to manipulate the natural state of the solventogenic bacteria, the ways in which they select, transport and ferment each type of sugar need to be fully understood. Since it is known that the PTS plays a central role in CCR, it is important to understand how the many PTSs present in a bacterium function, what each one of them transports and how they may be related to control of sugar metabolism.

4.1 Bioinformatic Analyses:

It has been determined through bioinformatic studies that *C. beijerinckii* has 43 separate and complete PTSs. Of these, three have been successfully analysed to determine which sugars they transport and phosphorylate. One operon (containing genes; *gut*A1, *gut*A2, *orf*X, *gut*B and *gut*D) was found to encode a glucitol PTS (Tangney et al, 1998), while Reid et al (1999) identified a system responsible for transporting sucrose (*Scr*ARBK). Most recently, it has been shown by Al Makishah and Mitchell (2013) that *cbei* 4532 and *cbei* 4533 encode a PTS that transports N-acetylglucosamine and glucose.

In this study, the amino acid sequences of the IIC domains of the 19 PT-systems belonging to the mannose/fructose/sorbose family were used to construct a phylogenetic tree and were compared for similarities. The IIC domains were divided into three separate branches (Figure 3.1). Of the systems studied in this project, Cbei 0712 was found to be most closely related to ManM, the IIC domain of the glucose porter in *Lactobacillus casei* (77% identity) and also shared 74% identity with the glucose/mannose system of *Listeria monocytogenes* and 67% with *Oenococcus oeni*. As for Cbei 3872, it was most almost identical to Cbei 4912 and showed 71% identity to PTFC, a fructose porter in *Bacillus subtilis*. Systems Cbei 0957 and 0965 were most closely related to one another, and clustered in a phylogenetic branch with Cbei 2907, Cbei 2901 and Cbei 2196. By identifying these relationships, some aspects of substrate specificity can be predicted. However, systems of the man/fru/sor family are known to exhibit a broad specificity, so the only way to identify the function of each system is by experimental analysis.

Shi *et al* (2010) analysed the phosphotransferases of the man/fru/sor family in *C. beijerinckii* by comparing the IIB domains, and suggested that they are responsible for the sugar specificity. However, these domains do not interact with the substrate. The IIAB domains of the four *C. beijerinckii* systems studied here showed a lower relationship to each other compared to the IIC domains, suggesting that they have different evolutionary pressures. In terms of analysing the function of the PTSs, it seems sensible to study the properties of the IIC (and IID) domains, which do actually interact with substrate molecules.

Since the investigated systems belong to the man/fru/sor family, they all comprise four EII domains, including a D domain. While it was attempted to clone all four domains for all four systems, there were reasons why only the IIC/D domains were cloned in some cases. In the case of system Cbei 0711-0713 and Cbei 3871-3874 all four domains were successfully amplified by PCR. All four domains of systems Cbei 0955-0958 and Cbei 0963-0966 were also successfully amplified using PCR, but only PCR products containing just the IIC/IID domains were successfully cloned into SoloPack cells.

The *in vitro* part of this study investigated the transfer of the genes for the IIC/D domains of systems, Cbei 0955-0958 and Cbei 0963-0966 and all four domains of systems Cbei 0711-0713 and Cbei 3871-3874 to a mannose-negative mutant of *E. coli.*

4.2 The PTS encoded by genes *cbei* 0711 and *cbei* 0713:

The first genes from *C. beijerinckii* to be amplified by PCR were *cbei* 0711-0713. These genes encode all four domains of a PTS belonging to the mannose/fructose/sorbose family. In this case, the mutant *Escherichia coli* ZSC113was used as it is unable to uptake mannose and glucose. The amplified genes were inserted into the plasmid cloning vector pSC-A-amp/kan before being transformed into *E. coli*. Four of the colonies found to contain insert were then chosen for plasmid extraction. One of the four plasmids was found to have insert in the reverse orientation, whereas the other three had insert placed in the forward direction, the favourable orientation for expression. A plasmid containing an insert in the forward orientation was selected and transformed into the mutant *E. coli* in order to investigate the fermentation phenotype of the PTS. Colonies that contained plasmid following the transformation were spread onto MacConkey agar containing mannose next to the untransformed mutant for comparison. While the results from the first transformation were unreliable due to poor growth of transformed cells, the second showed clear negative reactions from all transformed colonies. The results were different to those of Lennon (2010), who isolated transformants that gave positive fermentation results.

Occasionally, mutated strains of *E. coli* revert to their natural state. If this were to happen in *E. coli* ZSC113, it would be able to ferment mannose and/or glucose like the wild type

strain. However, while a reversion is possible, it is uncommon in this strain and therefore not the likeliest explanation for Lennon's results.

Once the fermentation phenotype of the transformants had been confirmed, transformed cells were grown in LB with 25mM of either mannose or glucose added. Growth and sugar utilisation were recorded over a period of eight hours and compared to the growth and sugar uptake of the untransformed mutant. The concentration of mannose decreased on average from 26.42 mM to 21.18 mM (-19.8%) while the concentration of glucose only decreased from 28.45 mM to 25.5 mM (10.3%). Although small, the reduction of sugar concentration was greater than seen for the control strain *E. coli* ZSC113 (-4.2%).

Similar experiments by Essalem (2014) investigating the utilisation of glucose and following the transfer of *cbei* 0751 an into *E.coli* ZSC113 showed that clones which tested positive for glucose and mannose fermentation greatly decreased the concentration of glucose and mannose in LB over 8 hours (59% and 57% respectively) while the control only decreased by 3.7%). While the decrease in mannose concentration for clones containing *cbei* 0711-0713 was rather higher than the control, the results were nowhere near to the decrease shown by these earlier results.

As the results gathered show no solid evidence of the system encoded by *cbei* 0711-0713 having the ability to utilise mannose, it can be concluded that this system is not responsible for mannose transportation. However, as the decrease in mannose was rather higher than that of the control, it may be that this system can transport mannose to a very limited extent.

4.3 The PTS encoded by genes *cbei* 3871 to *cbei* 3874:

The second set of genes to be amplified encoded Cbei 3871-3874. This set included the domains A, B C and D of Enzyme II, and therefore encoded the complete PTS and should have had a greater chance of complementing *E. coli* ZSC113. However, interaction between the *E. coli* and clostridial PTS must still occur, at the level of transfer of phosphate from the HPr to IIA domain. The genes were successfully amplified by PCR and inserted into cloning vector pSC-A-amp/kan. Three of the colonies that resulted from the cloning were shown to contain the insert and so plasmid was extracted from them. Two of these plasmids had inserts in the reverse orientation while one was shown to be in the forward orientation and was used to transform *E. coli* ZSC113.

The resulting colonies were tested for fermentation phenotype on MacConkey agar and all were found to be negative on agar containing mannose or glucose. Transformed cells and untransformed cells were again compared for growth and mannose/glucose uptake in LB over an eight hour period. While both cell types grew substantially, the levels of mannose and glucose did not decrease greatly over the 8 hour period. Mannose levels dropped from 26.56 mM to 23.74 mM (-10.6%) and glucose dropped from 28.85 mM to 24.68 mM (14.65%).

The system encoded by *cbei* 3871 to *cbei* 3874 consistently showed no strong ability to transport mannose when transformed into the mutant. As the transformed genes contained the entire Enzyme II, its lack of ability to transport mannose could not be due to the fact that any EII domains were missing. The fact that both mannose and glucose levels dropped by a small extent in the growth tests, might indicate that the system allows small amounts of mannose and glucose to be transported, but neither would appear to be the sugar that the system is designated for.

4.4 The PTS encoded by genes *cbei* 0957 and *cbei* 0958:

The third part of the investigation was to study the effects of the system encoded by *cbei*0957 and *cbei*0958, when transformed into the mutant *E. coli* strain. The genes do not encode a complete PTS, only the IIC/D domains, and so would be reliant on a functional Enzyme I, HPr and Enzyme IIA and IIB domains from the host species.

The two genes were amplified by PCR and inserted into the cloning vector. Unlike the cloning stage of the other systems which produced several colonies containing insert, only two colonies were shown to possess plasmid containing insert on this occasion and so were both screened for their orientation. One was shown to be clearly in the reverse orientation whereas the other gave an ambiguous result, producing a band in both screening reactions. Since the intensity of the amplified DNA band was greater for PCR using the M13 reverse and reverse cloning primers, it was assumed that the insert in this plasmid was in the preferable orientation. Transformants of *E. coli* ZSC113 were tested on MacConkey agar and all colonies showed a negative fermentation phenotype. These transformed cells were also tested for their ability to utilise mannose and glucose in LB over a period of eight hours. The concentration of mannose at the start of the experiment was recorded as 28.84 mM which dropped to 27.1 mM by 8h (-6%). The change in glucose concentration was even less, starting at 30.85 mM and dropping to 29.64 mM

after eight hours (-3.92%). As the change in sugar concentration was so slight, it distinctly shows an inability to transport both mannose and glucose.

Since the genes transformed into the mutant encoded only the IIC and IID domains of the protein, it was not entirely clear as to whether the transformants showed no ability to ferment mannose simply because the PTS system could not transport it or whether the IIA and IIB domains were absent and the *E. coli* PTS could not interact with the clostridial domains.

4.5 The PTS encoded by genes *cbei* 0965 and *cbei* 0966:

Finally, the genes encoding PTS proteins Cbei 0965-0966 (encoding IIC and IID domains only) were investigated. *E. coli* ZSC113 colonies transformed with either of the two plasmids containing the insert showed positive fermentation results when spread onto MacConkey agar containing mannose, but not when spread onto those containing glucose as the carbon source. When these colonies were respread onto MacConkey plates containing mannose or glucose and without kanamycin, to be compared to the untransformed mutant, they produced the same results while the mutant continued to show negative results on both sugars. This suggests that the PTS encoded by *cbei* 0963-0966 can indeed transport mannose. In order to fully investigate this, a few additional experiments will need to be carried out. Results from growth experiments whereby transformed cells are placed in LB containing either mannose or glucose over a period of eight hours as done with the other systems would have indicated whether or not the transformants were indeed able to uptake mannose. Unfortunately there was not enough remaining time in the laboratory in order to conduct these experiments.

4.6 Mutations in *E. coli* ZSC113:

It was decided to sequence the mannose PTS (ptsM) of *E. coli* ZSC113 in order to determine where the mutations lie within the genes, so that it could be deduced which domains of Enzyme II had been mutated. In determining which domains had undergone mutations, it could be decided how valuable the results gathered were if only IIC and IID domains had been transformed into the mutant. If the IIA and/or IIB domains could not function, this might have explained why *cbei* 0711 to *cbei* 0713 and *cbei* 0957/ *cbei* 0958 gave negative results.

The DNA sequence of the mannose PTS was compared to that of the wild type *E. coli* K-12 obtained from the Colibri website (http://genolist.pasteur.fr/Colibri/). A total of five mutations were found; one in *manX* (encoding IIAB) which replaced a C with T, three in *manY* (encoding IIC) which consisted of a change of two T bases to C bases and one A base to a G and one found in *manZ* (encoding IID) which had a G changed to an A. This meant that all Enzyme II domains contained at least one mutation.

The most significant finding was that *E. coli* ZSC113 contains a nonsense mutation in the *manX* gene, meaning that functional IIA/IIB domains will not be present in the cell. This could have explained why the transfer of *cbei* 0957– 0958 (IIC and IID domains only) did not allow the uptake of mannose. However, the addition of *cbei* 0965 and *cbei* 0966 (also IIC and IID domains only) did allow *E. coli* ZSC113 to uptake mannose, suggesting that the IIA and IIB domains from another PTS may have been able to complement the proteins produced by *cbei* 0965 and *cbei* 0966, but not the others. There were three mutations in the IIC domain which either consisted of a change in codon, but encoding the same amino acid or a substitution for another amino acid. The first mutation found in *manY* changed a T base to a C base (GGT to GGC) at codon 40. Both codons encode cysteine and so this mutation would have caused no changes to the protein produced. The second mutation was also a substitution of a T base to a C base (AAT to AAC) at codon 58. Both codons encode asparagine and so this mutation would not have made any changes to the protein. The third changed an A base to a G (ATT to GTT) at codon 153. This would have lead to a substitution of isoleucine to valine. It is currently unknown exactly how this substitution would affect the protein produced, but both amino acids are non-polar with a neutral pH and so the changes may be minimal. There was one mutation found in the *manZ* gene (IID domain) at codon 20 which replaced a G base for a C base

(GGC to GAC) causing the codon to encode polar aspartic acid instead of the non-polar glycine. It is currently unknown exactly what affect this change has on the final protein.

According to Shi *et al* (2010) the IIB subunit is the one which decides the substrate specificity of *C. beijerinckii* man/fru/sor phosphotransferases. However, since the introduction of genes *cbei* 0965 and *cbei* 0966 (IIC and IID domains only) allowed the transformed *E. coli* ZSC113 to ferment mannose, this would suggest that substrate specificity is actually dependent on these subunits, which interact directly with the substrate.

A collection of *E. coli* K12 mutants known as the Keio collection whereby 3985 genes were systematically deleted and replaced with a gene encoding resistance to kanamycin exists (Baba et al, 2006). This collection contains mutants with knockouts disabling individual PTS proteins, including those of the mannose PTS. Analysis of complementation of these specific mutations may be useful in future investigations of the *C. beijerinckii* man/fru/sor PTS phosphotransferases.

4.7 Further Investigating the PTSs of *C. beijerinckii*:

Assuming that all cloned clostridial *pts* genes were expressed in *E. coli*, the results obtained from each of the fermentation experiments suggest that the phosphotransferase systems Cbei0711-0713, Cbei3871-3874 and Cbei0955-0958 do not transport mannose or glucose. As these systems are a part of the mannose/fructose/sorbose family, it could be that these systems transport fructose, sorbose or another similarly structured hexose sugar. PTSs which are members of this family are known to have a broad substrate specificity, often transporting several related sugars. From their bioinformatics analysis, Shi *et al* (2010) concluded that all members of the family in *C. beijerinckii* are fructose transporters. In order to investigate this, a mutant bacterial strain which cannot transport fructose would be required. All major fructose transportation routes would have to be disabled to create a fructose transport-negative mutant.

There are a number of routes by which fructose can be utilised in wild type *E. coli* (Kornberg, 2001). According to Kornberg, the route which predominates all others in wild-type strains is encoded by the fructose operon. This operon contains genes encoding a membrane-spanning PTS protein (FruA containing IIB and IIC domains), a 1-

phosphofructose kinase (FruK) and a diphosphoryl transfer protein (FruB) incorporating a fructose-specific HPr domain and an EIIA, and is under negative regulation by a *fruR* gene. Mutating the *fruA* gene would therefore be essential to prevent fructose uptake. Fructose is also transported by the mannose PTS (ManXYZ), by the mannitol PTS (MtlA) and the glucitol PTS (GutA), but the latter two must be greatly overexpressed in order to transport fructose effectively. Mutants lacking the PTS-dependent routes of the fructose uptake can utulise fructose by diffusion via an isoform (PtsG-F) of the major glucose PTS and then phosphorylation by ATP and a mannofructokinase (Mak+). However, this route is only relevant in strains lacking the fructose phosphotransferases and is dependent on a point mutation within the PtsG-F protein.

As *E. coli* ZSC113 already lacks an operative mannose PTS, it can be expected that inactivating the *fruA* gene in this strain would produce a fructose-negative phenotype. One possible method which could be used to create a fructose-negative mutatnt is via phage transduction of the *fruA* mutation of the *fruA* mutant of the Keiocollection into ZSC113. The newly created mutant could be selected for on MacConkey agar plates containing fructose and kanamycin, and used for complementation studies.

4.8 Final Conclusions :

Few transport systems in clostridia, let alone *C. beijerinckii* have been properly characterised. The characterisation of phosphotransferase systems in clostridia is important in fully optimising the ABE fermentation process. In this investigation no evidence was found to indicate that Cbei 0711-Cbei 0713 and Cbei 3871-Cbei 3874 can transport mannose or glucose significantly, although these systems may be able to transport the sugars at a low rate. System Cbei 0955-0958 also did not appear to transport either mannose or glucose whereas Cbei 0963- Cbei 0966 was indicated to be a transporter of mannose. By using mutant strains which lack the ability to uptake other hexose sugars such as fructose, it can be determined whether these systems are responsible for transporting alternative sugars. The mutant strain of *E. coli* ZSC113 which lacks the ability to uptake mannose and glucose was isolated 40 years ago following chemical mutagenesis, but until now the exact location of the mutations affecting PTS activities were unknown. Sequencing of the mannose PTS and comparing the results to the wild type showed mutations in all three proteins making up the Enzyme II. However, the most critical mutation is in the *manX* gene because it is a stop codon that will terminate synthesis of the protein prematurely. Now that knowledge of the exact mutations of the *manXYZ* genes has been obtained, it would be worth revisiting the work this time complementing specifically with IIA and IIB domains rather than with the IIC and IID domains.

5. Appendices

Appendix I: Gene sequences

Clostridium beijerinckii NCIMB 8052 (*cbei* 0711-0713)

ATAGGGTATTATCATATTTTTTTATGATTTTAATTTTGGTGTACAAATATTGTGACGATA
TACGAATATATACTATTGTTTTATTTCCAAATGCTTATGCCTTTTGTTTTTGTAACAAAT
AAAAAATAGGAGGTATAAAAATGGTAGGAATTATTCTTGCTAGTCACGGAGAATTTGCTA
AAGGCATCATGCAATCTGGTGCGATGATTTTCGGAGAACAAGAAAACGTAAAAGCTGTTA
CGTTGATGCCTAGCGAAGGACCTGATGATCTTAGAGCAAAAATGAAAGATGCAATCGCAT
CCTTTGACAACCAAGATGAGGTTTTATTCTTAGTTGATCTTTGGGGTGGTACACCATTCA
ACCAAGCGAATATGCTATTTGAAGAACATAAAGATAAATGGGCAATCGTAGCTGGTTTGA
ATTTACCAATGCTGATTGAAACTTATGGTGCACGTCTTTCAATGGAATCTGCTCATGAAA
TTGCAGCTTATATCTTAAATGCAGGTAAAGAAGGAGTTAAAGTTAAACCCGAGGAGTTAG
AACCAGCAGATACTGGTAATGCTTCAGGAGCGGGAGCAGGGCAATCTAATGCAGGTGCAC
CTGGATCGTTTGAATACGTTTTAGCTCGTATCGACTCTCGTTTACTTCATGGTCAAGTAG
CAACTGCTTGGACAAAAACTGTAAATCCTACACGAATTATTGTCGTGTCAGATGATGTAG
CTAGAGATACTCTTCGTAAGAATTTGATTACGCAAGCTTCTCCTCCGGGGGTTAAGGCTC
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GCAAAGTTTTAGCTTTCAATCAAGAAGATATTGATATATTCAATAAGCTTAAACAAGCTG
GACTTACTTTTGATGTGCGTAAAGTACCAAATGATTCAAAAGCAAATATGGACGAAATAC
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TACTGGTGATCCTTTAGGGGATCTAATAGATAGATACTAAGAAGGGAGAGGAAACGAAAA
TGTCAAAAGAATTAAAATTAACAAAAAGAGACCGTATTTCTGTTTGGTTCCGTTCATTTT
TCCTTCAAGGTTCTTGGAACTATGAAAGAATGCAAAATGGTGGTTGGGCATTTGCAATGA
TTCCAGCAATCAGAAAATTATATAAGACTAAAGAAGAGAGAGCTGCAGCATTAGAACGTC
ACTTAGAGTTCTTTAACACTCACCCATATGTAGCTTCACCAGTTGTTGGTGTAACATTAG
CTTTAGAAGAAGAACGTGCAAATGGTGCACCAATCGACGATGTAACTATTCAAGGTGTTA
AGATTGGTATGATGGGACCTTTAGCAGGTATTGGAGATCCAGTTTTCTGGTTCACTGTAA
GACCAATTTTAGGAGCATTAGCTGCTTCACTTGCTCTAGGTGGTAACATCCTTGGACCAA
TTATCTTCTTCTTCGCTTGGAATATCATCCGTATGGCATTTATGTGGTATACACAAGAGT
TTGGTTACAAAGCAGGATCTCGTATTAGTGAAGATTTATCAGGTAATATGCTACAAGATA
TTACAAAAGGAGCATCTATCCTTGGTATGTTCATCTTAGGATCGTTAGTTAACAGATGGG
TATCTGTTAAATTTGCACCAGTAGTATCATCTGTTAAATTAAGTGATGGTGCATTTATTG
ATTGGAGCAAACTTCCTGCTGGAGCAGAAGGTGTTAAGCAAGCTCTATTACAACAAGCAT
CAGGTATGTCATTAACTGATACTAAGATTACAACATTACAAAATAACTTAGATTCATTAA
TCCCTGGATTTGTAGGATTATTAATTACACTTCTTTGTATGTGGTTACTTAAGAGGAAGG
TATCTCCAATTCTTATAATTCTTGGATTGTTCATAATTGGTATTGTTTTCCACTTGATCG
GTTTAATGTAGTACTTTTTTACTTAGCCTAGGCTTTTTAGCCTGGGCTTTTGTTAACAAT

ATTAATCGTGTTACTTTTAATAGACTTTGGAATAATTGATAAAAGCATGTAAAGAATATA

Figure 5.1: The sequence of genes *cbei*0711-0713 and annealing positions of primers used.

Key:

Forward primer 396 annealing site

Forward primer 397 annealing site

Reverse primer 398 annealing site

Clostridium beijerinckii NCIMB 8052 (*cbei* 3875-3870)

TGAAGCTTGTTTTGGTGTAGGAATTTAACTAAAACATTAAATTTAAATTAGTATATATGT
TGTGATAATAATTTTAAGCTTAAAACTCCATTAAGCTGAGTGCTCTATTAAAACTAGGAT
TTACAAACTTAATCGCAATATATAAAAAATTATGACTTAAAAATACGAAATATAAAGGTT
AACTGCCATTAATACATGGATCTAAAAGGATCGTGTGTTAGTGGCAGTTAATTTTTATAT
ATATGTGTGTTAATTCTACAAAATTATTTAATAAACTGTGTTAAATGTGATTTGAAGTAT
GTAAATCTTAAATTCATAGTGTAATCAAAAATAGTTAAGTATCAGAAAAGGTAGTTATAG
CCTTATTTCTTGATGATTTTTTTATTTACATCCTTAGTTGGCATAGGAATTGCTATGTAT
ATAAATATAAAAATAATATATGTAGCAAGAAAGGATGATAAAGTAATGATCTCAGTAATT
ATAGGTACCCACGGTATATTTTCAGAAGAGATTCTTAAGTCAGCAGAAATGATTTTTGGA
ATTCAGGAAAATGTTGGTGCCGTTACATTTAAACCAGGTGAAGGAATTGAATCACTTGTA
GAAAAATATAACACTCTTATAAAAGAGTTAGATTCAACAGACGGAGTACTATTTATGGTA
GACCTGTTTGGAGGAAGTCCATTTAATGCGGCAAGTATAATTGCAATGCAACATGAGAAT
ATGGAAATTGTAGCAGGTGTTAATCTGCCAATGATTTTAGAGGTTCTAGGAAGTAGAGAT
TTTTCAAGCATAACAGAATTGTTAAGAGTAGCAGAAAACTCTGGAAAAGAGGCTATAAAG
GTTTTAACTAAAAATATCAATATAGATGAAGATGAAGAAATTATATAAGTATTTCAGATA
ACTGCATTTTTAGTTCCATAATTATAATAAGCAAAGTTAAGTGAGAATACGAATACATAA
GTTCTTTGGAAAATAATATTGAAAATTTATGCAACAAGTTATATCAATATAAGCGCAATT
TAAGTAAATATAAAAATTAGAAGATAGGAGATAAAAAATGGAAATTAGCTTTGTAAGAAT
AGATGACAGATTAATACATGGACAAGTAGCAACAGTTTGGACTAAGGAGAGTGGGTGTAA
CAGAATATTTGCCTGCAGTGATGAAGTTGCAGCGGATGACTTAAGAAAACAATTAGTAAT
TCAAGTAGCTCCTCCAGGGATAAAAGCATATGTGCTTCCAATAGCAAAGGCAATTGAAGC
ATATAATAATCCTAAATTTGACAGCTTTAAAACTTTATTTTTATTTACAAACCCTACAGA
TGTATTAAGAATGGTTGAAGGCGGAGTACCTATTAAATCTGTAAACGTTGGAGGAATGTG
CTATAAGGCTGGTGATAAACAGGTTACAAATGCATTATGCATGAATGATATTGATATTGA
GGCGTTTAAAAAGCTTAATGAAAAAGGTATCGAATTAGAGGTAAGGAAACTAGCAAAAGA
TACTAAGATAAACTTAATGGACAGATTAAAGGAATTACAATTTATATAAGTATATAAAAA
CTTTTTTATATAAATCAAAAAAAGAAAGAGAGGAATTATAAATGAGCACTTTACAATTAA
TTTTATTAGTAATAGTTGCAGCAATTACTGGTATGGCAAGTGTACTTGATGAGGCACAGT
TCCACAGACCAATAATTGCATGTACACTAGTAGGTCTTGTACTAGGAGATATTAAAACAG
GAATTATACTAGGTGGAACGCTTGAAATGTTAGCATTAGGATGGATGAATGTTGGTGCAG
CTATGGCACCAGATGCAGCGCTTGCAAGTGTAATTTCAGCAATACTTGTCATAATTGGTA
AACAGTCAATTGGAGCAGGTATAGCAGTTGCGGTTCCAATAGCAGCAGCAGGACAAGTTC
TTACTATTTTCGTAAGAACAATAACAGTGTTCTTCCAACATTTAGCTGACAAATATGCTG
AAAAGGGTAACACAAGAGGAATAGAAATGTGTCATATTCTAGGTTTATCACTTCAAGCAA
TACGTGTAGCTATTCCAGCTGCAATCGTAGGTGTACTTGCAGGTACAGATGCTGTAAATG
CAGCACTTGCAGCTATACCACAAGTTATTACAAGAGGGCTTCAAGTGTCAGGTGGATTTA
TAGTAGTTGTTGGTTATGCAATGGTAATGAATATGATGAATAGTAAATCATTACTACCAT
TCTTCTTTATCGGATTCTTGTTAGCAGCATTTACAAACTTCAATTTAATTGGTTTTGGTG
CTGTAGGTGTTATTGCAGCAATATTCCATATAAAATCAATAGCTAATGAAGGTAAAGTAG
CAGTAGCTGGAGCTGGAAGTATTGATGATATCGATGATTCAGACCTAATGTAATTACAGA
AAGGAGATAAAATAATGAGTGAAAAGAAATTAAATAAAAGTGATATAGTAAAAATGTTTA
TCCGTTCAAATTTCTTATTGGGATCATTTAATTTTGAAAGAATGCAAGCAATAGGTTTTT
GTGTTACATTAATTCCAGCTTTGAAGAAATTATATAAAGGTGACGAATTAAGTGCAGCAC
TTAAAAGACATTTGGAATTCTTTAATACACAACCATTTATGGCAACGCCAATTATGGGAA
TAACAGCAGCTATGGAAGAACAAAAGGCAAATGGGGCAGATATAGATGAGGCATCAATAA
GTGGTGTTAAGATTGGTCTTATGGGACCACTTGCCGGAGTTGGAGACCCAATATTCTGGG
GAACACTAAGACCTGTGCTTGCAGCATTAGGGGCAGGACTTGCACTTACAGGAAGTATAA
TTGGACCGCTAATTTTCTTCATAGGATTCAATGCAATAAGACTTGCAACAAACTGGTATG
GAATGTTCTATGGATATGAAAAAGGAACTCAATTAGTTGCTGATATGGGAGGAAATAAAC
TTAGATATCTTACAGAAGGTGCTTCAGTACTTGGATTACTTGTTATAGGTGGTCTTGTTT
CAAAATGGACAACTGTTAATATACCTTTTGTTCTCTCTAAATATACACAAGCTGATGGAA
AAGAAGTAGTTACAACTATTCAAAGTGTACTAGACAGCTTAATGCCAGGATTAGTTCCAC
TTTTACTAACATTTTTATGTATGTATCTATTAAAGAAGAACGTGAATCCATTATTAATAA
TCTTTGGATTGTTCGCAGTAGGGATTTTAGGAGTTGCGTCTGGAATATTACAATAAACAT
TAATTCATAGTCTTATTTTAAGTAAACCATTTTAAATTGAAATTTATTATAGCAGTATAG
ATTACCTAATTAATATTATAAATTAAAGGTCTAACATTAATTAAATTAGTAGGGTAGTCT
ATACTTGTATTAAAAACAATATTCTCATGATTATATAGTGAATTTAATCGTAAAGTAAAC

GTTACCTAAGGCAGAGGAGTTCATATAGTACAGTAAATTCATATGAAAGTACAAATTTAA
AAGTACTAAAATAATTATATTTATAGAATCAAAGGATAAACACTTCTAAAAAATAATGAT
AAAAGGGGTAAAAAAATG

Figure 5.2: The sequence of genes *cbei*3875-3870 and annealing positions of primers used.

Key:

Forward primer 700 annealing site

Forward primer 701 annealing site

Reverse primer 702 annealing site

Clostridium beijerinckii NCIMB 8052 (*cbei* 0954-0959)

TAAGTGATAGAGATAGTGAAGAATAAAGAAGCGTGCCAGACATTGGCACGCTTCTTGCTT
TTATAATGCTATGAGAAGTGAAATAAGATTTATAATATCATGAGTTTGGATGGAGATGAT
AAATTTTGAAGAGAAGATTTTTATTAGCAAGTCACGGAAGACTTGCAGATGGAATGCATG
AATCTGTAAAGATAATAATAGGAAAACAAGATAATATATCAACACTTTGTGCATACATAA
GCAGCGAAAAAAATTTAAGCCAACAAGTAAAGGAGGTCATAAATAATTTAGAAGATGGGG
AAGAGTTAATAGTAATAACGGATATCTTCGGAGGAAGTGTAAATAATGAGTTTATGAAAT
ACATTAATTATGAAAACTTACACATTATTTCGAGTATGTGTTTACCACTTGTAATTGAAT
TAATAACAAGCCAAGAAGAGAGTACAGAAAAATTAATCGAAGAAACAATTAAAAATACGC
AAGAGAATATAAGATATTGTAATAAAACATTTCATACGGCTAAATCAATTAGAGACGAAG
AATTTTAAAGGAGTGATTTTATATGATTAAACTATTAAGAGTAGACTATCGTTTGTTACA
TGGACAAGTGGCATTAGCATGGACTCAACAAATTAACACAGATTGTATATTAATTGCAAA
TGATAAACTTATGCAAGATGAGATAAAGAAGACAACAATAAAGTTGGCTAAACCTAATGG
GGTTAAATTAGTAATTAAGAATATAGAGGATTCCATTGCGGCTATAAAAAGTGGAGTTAC
AGATAAATATAAGTTATTCATTATAGTAGAAACAATAGAAGATGCAGCAAAATTAAGCAA
TGAATTGCCTGATATAAAATCAATTAATATAGGAGGTGTTATGCCTAGAGAAGGGACAAG
AACAATCACTAAATCAGTTCCAATATCAAAGGAGGAGGAAAGAATATTACAAGAACTAAT
AAATAGAGATATTGAATTAGAGATAAGACAGGTTCCAGGTGATAAGAAAATTTTAGTTAA
AAACGTATTATAAGGAGGTAAAATTGTATGACTCAAGCTATTTTAGTAGGATTGATTGCA
ATGTTTGTTACGTTTGAATGGTGTTTAGGATCATGCTTAATAACAAGGCCAATAGTAACA
GGAGTACTTGTAGGATTAGTTATGGGCGATTTACAAACAGGTATTATAATGGGAGCAACT
CTTGAAATGGCATTTATTGGAGCAGTTACATTAGGTGCAGCAGTTCCACCAGATGTTATA
ACAGGAGGGATCTTAGGTACAGCTTTTGCGATAGCAACAGGGCAAGGAGCAGAAGTTGCA
TTGACATTGGCATTTCCAATAGCAACGCTATTTCTATTAGTTGATAATGTTATGACACTT
TTTGTAATGCCATTTTTTCTTCATAAAGCAGATAAATATGTAGAAGAGGGAAATTTCAAC
GGTGTAAGTAGGATGCACTTTTTAGGGGGATTTGTAGTAAAGAGCTTACCAAGAGGAATT
TTTGTTGCACTAGCATTATATTTAGGAAGCCCATTTATGAGCGCCGTTCTAGATAGCATT
CCACAATTTGTACAAAATGGGCTAGTAGTTGCAGCAGGATTTATTCCAGCTTTAGGAATA
TCACTTCTAGCACAAATGATTTTGACAAAAAAATTGGTTATATTCTTTGTATTAGGATTT
GCAATTAGTGCATATTTAAAAATACCTATGCTAGGTGTTGCAATTTTTGCAACTATCATT
GCGATAGTTCTTGTGGGATTACAAAACAATAATGTAAAGAAACAGGAGGTAATGCTAGAT
GAAGATTTCTAATAATTTAGAACAATCAAATGATGTTAAAGTTACTAAAAATGATCTTAA
GAAGATGTTTTTGAGAGGCCTTGCTTTAGAATATTCATGGAATTATGAACGCCAACAAAA
TATGGGGTATTGCTATGCTATGCTGCCGATTATTAAGAAACTTTATAAGAAAAAAGAAGA
GCAAATAGAGGCCGTAAAAAGGCATATGGAATTCTTCAATACAACACCATATGTATCAAC
TTTAGTATTAGGGATATCTACAGCAATGGAGGAGAGTAACGCAAATAATGAAGATTTTGA
TACATCTTCAATAAGCAGCGTAAAGGCATCTCTAATGAGTCCCTTAGCAGGAATAGGAGA
TTCGCTTTTTTGGGGAACATTAAGGGTAATAGCTACAGGAGTTGGAACATCATTATCATT
ACAAGGAAATATTTTAGGACCAATATTGTTTTTACTATTATTTAATATACCACAATTTAT
TGTTAGATATGTCTGTATGATAAGTGGATATAAATTTGGAACGAAGTTTCTTGAAAAAAT
AGAAGACTCTGGATTAATGCCTAAGTTAACATATGGGGCTTCAATAATTGGATTAATTAT
AATAGGAGCAATGATACCTGGAATGGTGAACATTAAAATTGCTGGAATGATGGGAACTGG
AGATAGTGCTGTTGAAATACAGAAGATAATAGATGGAATAATGCCATATATTCTTCCTCT
TGGGCTTACTACTATAATTTACTCATTATTGCAGAGGAAATTAAAAGTTATATATATACT
AATAGGATTAATGGCATTAGGCATATTAGGCACATTTTTAGGAATATTTGCAGCTTAATA
TTAGAGATGCAATTATATAAATATTTATTACGTAGATATCAATATATAAAAATTAGGAGG
ATATTATG

Figure 5.3: The sequence of genes *cbei* 0954-0959 and annealing positions of the primers used in this project.

Key:

Forward primer 703 annealing site

Forward primer 704 annealing site

Reverse primer 705 annealing site

Clostridium beijerinckii NCIMB 8052 (*cbei* 0962-0967)

ATGATTTCAAGCGATTAAGAGAATACAATAAAGTTTTATAAAAAGCATGTCACTAGTTGGC
ATGCTTTTTGCTTATTAATAAGGTATAAATAAAAAAACAAAGGAGGTGATGGTAATGAAG
AAATTTCTAATTGCAACTCATGGAGAACTATCAAAAGAATTTATAGAAACTAGTAAATTA
ATAGTAGGATCATTAAGCAATGTTGAATATTTCTGCATGACTAAAGATAAATCGGGAGAT
GATGCAGAAAAGGAAATGAAAAGAATTTTATCTAATAAAAGCGAAGAAGAAAATTATATT
GTTTTAACAGACATTTTTGGTGGAAGTGTAGCTAATTTATGTACCAATTTATTGCTAAAT
GGATATGAATTTGAATTAATAACAGGTATAAATTTGCCAATGATTTTAACAATACTATTA
TCAGATGAAGAGGATATAGAAACATTGGTAAAGAACGGAATAGAAGAATCAAAAAAAGGA
ATTATTCATGTAAATGAATTACTAAAAAACAATAATGAAAGGAATGAAGAAGATGATATC
ATTGATGAGAATTGACGACAGATTAATACATGGTCAGGTTGCATTTGGATGGTCAACATC
ATTAGGAATAAATGTAATATTAGTAGTTAATGACGAAGCAAAAAATGATCAAATGAAGGC
CATGGCATTAAACTTAGCTAAACCAAACAATGTAACTTTGTATATTAGGGGAGTACAGGA
ATCAGGTGAGATAGTAGAGAAATTTGCTCAATCTAAAAAGAATAATGTACTTGTTTTAGT
GAAAAATACAAAAGATGCATTGGAATTAGTAAAAAATTCAGGCGGAGCTATAAAAGAGTT
AAATGTAGGGGGCTTGAGATATGAAGCTGGAAAGCAGAAGCTAACAGATTTAGTAGCAGT
TGATGAAAATGATGTATCTAATTTAAAAGAAATAAATGATTTAGGCATAGAAATTGAATT
TAGAATGCTGCCAAGGGATAAGAAAAAGACTTTATCAGATTTACTAAAATAAGTAAGGAG
GTAAAAAATTATGATAGCACAAGCAATTATGCTGGGATTACTTGCAGGAATAGGTATACT
GGATGGACGTATTTTTGGGCAGATAATGCTTGAAAGACCAATAATCACAGGTGCCTTAGT
AGGACTAATTTTAGGCGATTTAAAAACAGGTATTATTATAGGAGCTCAACTTGAACTTAT
ATGGATGGGTATTGCAGGAATAGGCGCAGCAACACCACCAGATGTTGTAACAGGTGGTAT
ATTAGGAACAGCTTTTGCAATATTATCTGGAAATGGTGTTGAGGTAGCTCTTGCTATAGC
AGTACCAATTGCAGTTTTAGCACAATCATTAGGAGTATTAGTAAGAATAATAAATTCTTA
TTTTGCTCAAAGGGCAAGTGTATATGCTAAAAAGGCTGACTTCAGGGGTATTGCAATAAT
GATGTGGATTCCGGTTATGTTATTTTTCCTAAGCACTTTTATTCCAACATTTTTAGCAAT
TATGTTAGGGGCAAATGCTGTTACAGCATTGATAAATTCCATTCCAAAGATAATTTTAGA
AGGGCTTGGAATAGCAGGATCATTATTACCGGCTGTAGGATTTGCACTATTAATGGATAT
GTTACTTTCAAAGAAAATGGCTGTATTTTTCTTCATAGGATTTTTATTAGCAGCCTACGC
TAAATTAGATATAACAGCAATAGCTTTATTTGGAGCATGTGTTGCATTTATATTAAATAT
TTATATTAACACTAAAGAAAATAGCACAGCTAAGCAGGCAGTTGCAAGTAATTTAACGGA
AGGGGAGATTGACTTTGAATAAGCTAGGTAAAAAAGAGTTAAGAAGTGTATTTTGGCGTT
CATTCGCTCTTCAAGGAGCATTTAACTATGAAAGAATGCAGAATTTAGGCTATTGTTATG
CAATGTTACCAGTTATTAAAAAACTTTATTCAAAGAAAGAAGATCAAGCTAAAGCTCTAG
AAAGACATCTTGAGATATTTAATACAACCCCAGTAATTGTTCCGACAATACTAGGAATTA
CTGCTGCAATGGAAGAACAAAATTCAAATAATTCCGATTTTGATGAAAGCGCGATTAGTG
CAATAAAGACTGCTTTAATGGGGCCTTTAGCAGGAATAGGAGATTCGCTCTTTTGGGGGA
CATTTAGAATTATAGCAGCAGGAATAGGTGTTTCTCTAGCCAGTGAAGGGAATATATTTG
GACCAATACTATTCTTACTATTATATAATGTACCAGGTTTTGCTATTAGAATATTGGGAT
TAAAATATGGGTACCAATTAGGAGTTAACTCATTAGAGAAGATACAAAAGCAAGGATTAA
TGGAAAAAATAATGTCTATAGCAACAACAGTTGGATTATTTGTTGTTGGTGGAATGGTAT
CTACTATGTTAAAAATAAAAACTCCGTTGGTATTTAATTTAAACGGTGCAAAAGTAGTAG
TTCAGGATATATTAGATAAAATATTGCCAAACATGCTGCCACTATTATTTGCCTTCTTTA
TTTACTATCTATTAAAGAAGAAAGTAAGCATCACTAAAATAACAATAGGTGTTATAGTTG
GTGGAATAATATTGCATGCAATATGGCTATTATAATAAAAAATAAGAGAAAGAGGTAATA
AAAATGATGAGAATACAAGATTATATGTTAGAAACTCCAACTAAAATGAAGGAGATAGTC
TCAAAATCTGATGAATTATTTAAAGAAATAATAAAGGAAGATATAGATAAAATTATAATT
ACAGGATCAGGAACAAGTTATCATTCAGGGGTACAAGTACAAACTTATTTGCAAGGAATA
TTAGATGCTGAAGTTATAGCAATGTATCC

Figure 5.4: The sequence of genes *cbei* 0962-0967 and annealing positions of the primers used in this project.

Key:

Forward primer 706 annealing site

Forward primer 707 annealing site

Forward primer 708 annealing site

Appendix II: *Escherichia coli* sequences

Features:

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Query  1     CTGAATCGATTCGATTGTGGACGACGATTCAAAAATACATCTGGCACGTTGAGGTGTTAA  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  120   CTGAATCGATTCGATTGTGGACGACGATTCAAAAATACATCTGGCACGTTGAGGTGTTAA  179

Query  61    CGATAATAAAGGAGGTAGCAAGTGACCATTGCTATTGTTATAGGCACACATGGTTGGGCT  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  180   CGATAATAAAGGAGGTAGCAAGTGACCATTGCTATTGTTATAGGCACACATGGTTGGGCT  239

Query  121   GCAGAGCAGTTGCTTAAAACGGCAGAAATGCTGTTAGGCGAGCAGGAAAACGTCGGCTGG  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  240   GCAGAGCAGTTGCTTAAAACGGCAGAAATGCTGTTAGGCGAGCAGGAAAACGTCGGCTGG  299

Query  181   ATCGATTTCGTTCCAGGTGAAAATGCCGAAACGCTGATTGAAAAGTACAACGCTTAGTTG  240
             |||||||||||||||||||||||||||||||||||||||||||||||||| |||||
Sbjct  300   ATCGATTTCGTTCCAGGTGAAAATGCCGAAACGCTGATTGAAAAGTACAACGCTCAGTTG  359

Query  241   GCAAAACTCGACACCACTAAAGGCGTGCTGTTTCTCGTTGATACATGGGGAGGCAGCCCG  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  360   GCAAAACTCGACACCACTAAAGGCGTGCTGTTTCTCGTTGATACATGGGGAGGCAGCCCG  419

Query  301   TTCAATGCTGCCAGCCGCATTGTCGTCGACAAAGAGCATTATGAAGTCATTGCAGGCGTT  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  420   TTCAATGCTGCCAGCCGCATTGTCGTCGACAAAGAGCATTATGAAGTCATTGCAGGCGTT  479

Query  361   AACATTCCAATGCTCGTGGAAACGTTAATGGCCCGTGATGATGACCCAAGCTTTGATGAA  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  480   AACATTCCAATGCTCGTGGAAACGTTAATGGCCCGTGATGATGACCCAAGCTTTGATGAA  539

Query  421   CTGGTGGCACTGGCAGTAGAAACAGGCCGTGAAGGCGTGAAAGCACTGAAAGCCAAACCG  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  540   CTGGTGGCACTGGCAGTAGAAACAGGCCGTGAAGGCGTGAAAGCACTGAAAGCCAAACCG  599

Query  481   GTTGAAAAAGCCGCGCCAGCACCCGCTGCCGCAGCACCAAAAGCGGCTCCAACTCCGGCA  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  600   GTTGAAAAAGCCGCGCCAGCACCCGCTGCCGCAGCACCAAAAGCGGCTCCAACTCCGGCA  659

Query  541   AAACCAATGGGGCCAAACGACTACATGGTTATTGGCCTTGCGCGTATCGACGACCGTCTG  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  660   AAACCAATGGGGCCAAACGACTACATGGTTATTGGCCTTGCGCGTATCGACGACCGTCTG  719

Query  601   ATTCACGGTCAGGTCGCCACCCGCTGGACCAAAGAAACCAATGTCTCCCGTATTATTGTT  660
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  720   ATTCACGGTCAGGTCGCCACCCGCTGGACCAAAGAAACCAATGTCTCCCGTATTATTGTT  779

Query  661   GTTAGTGATGAAGTGGCTGCGGATACCGTTCGTAAGACACTGCTCACCCAGGTTGCACCT  720
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  780   GTTAGTGATGAAGTGGCTGCGGATACCGTTCGTAAGACACTGCTCACCCAGGTTGCACCT  839

Query  721   CCGGGCGTAACAGCACACGTAGTTGATGTTGCCAAAATGATTCGCGTCTACAACAACCCG  780
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  840   CCGGGCGTAACAGCACACGTAGTTGATGTTGCCAAAATGATTCGCGTCTACAACAACCCG  899

Query  781   AAATATGCTGGCGAACGCGTAATGCTGTTATTTACCAACCCAACAGATGTAGAGCGTCTC  840
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  900   AAATATGCTGGCGAACGCGTAATGCTGTTATTTACCAACCCAACAGATGTAGAGCGTCTC  959

Query  841   GTTGAAGGCGGCGTGAAAATCACCTCTGTTAACGTCGGTGGTATGGCATTCCGTCAGGGT  900
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  960   GTTGAAGGCGGCGTGAAAATCACCTCTGTTAACGTCGGTGGTATGGCATTCCGTCAGGGT  1019

Query  901   AAAACCCAGGTGAATAACGCGGTTTCGGTTGATGAAAAAGATATCGAGGCGTTCAAGAAA  960
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1020  AAAACCCAGGTGAATAACGCGGTTTCGGTTGATGAAAAAGATATCGAGGCGTTCAAGAAA  1079
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Query  961   CTGAATGCGCGCGGTATTGAGCTGGAAGTCCGTAAGGTTTCCACCGATCCGAAACTGAAA  1020
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1080  CTGAATGCGCGCGGTATTGAGCTGGAAGTCCGTAAGGTTTCCACCGATCCGAAACTGAAA  1139

Query  1021  ATGATGGATCTGATCAGCAAAATCGATAAGTAACGTATTGTGTTGATTATCACTCAGTTT  1080
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1140  ATGATGGATCTGATCAGCAAAATCGATAAGTAACGTATTGTGTTGATTATCACTCAGTTT  1199

Query  1081  TCACACTTAAGTCTTACGTAAACAGGAGAAGTACAATGGAGATTACCACTCTTCAAATTG  1140
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1200  TCACACTTAAGTCTTACGTAAACAGGAGAAGTACAATGGAGATTACCACTCTTCAAATTG  1259

Query  1141  TGCTGGTATTTATCGTAGCCTGTATCGCAGGTATGGGATCAATCCTCGATGAATTTCAGT  1200
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1260  TGCTGGTATTTATCGTAGCCTGTATCGCAGGTATGGGATCAATCCTCGATGAATTTCAGT  1319

Query  1201  TTCACCGTCCGCTAATCGCGTGTACCCTGGTGGGCATCGTTCTTGGGGATATGAAAACCG  1260
             |||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
Sbjct  1320  TTCACCGTCCGCTAATCGCGTGTACCCTGGTGGGTATCGTTCTTGGGGATATGAAAACCG  1379

Query  1261  GTATTATTATCGGTGGTACGCTGGAAACGATCGCGCTGGGCTGGATGAACATCGGTGCTG  1320
             |||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||
Sbjct  1380  GTATTATTATCGGTGGTACGCTGGAAATGATCGCGCTGGGCTGGATGAACATCGGTGCTG  1439

Query  1321  CAGTTGCGCCTGACGCCGCTCTGGCTTCTATCATTTCTACCATTCTGGTTATCGCAGGTC  1380
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1440  CAGTTGCGCCTGACGCCGCTCTGGCTTCTATCATTTCTACCATTCTGGTTATCGCAGGTC  1499

Query  1381  ATCAGAGCATTGGTGCAGGTATCGCACTGGCAATCCCTCTGGCCGCTGCGGGCCAGGTAC  1440
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1500  ATCAGAGCATTGGTGCAGGTATCGCACTGGCAATCCCTCTGGCCGCTGCGGGCCAGGTAC  1559

Query  1441  TGACCATCATCGTTCGTACTATTACCGTTGCTTTCCAGCACGCTGCGGATAAGGCTGCTG  1500
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1560  TGACCATCATCGTTCGTACTATTACCGTTGCTTTCCAGCACGCTGCGGATAAGGCTGCTG  1619

Query  1501  ATAACGGCAACCTGACAGCGATTTCCTGGATCCACGTTTCTTCTCTGTTCCTGCAAGCAA  1560
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1620  ATAACGGCAACCTGACAGCGATTTCCTGGATCCACGTTTCTTCTCTGTTCCTGCAAGCAA  1679

Query  1561  TGCGTGTGGCTGTTCCGGCCGTCATCGTTGCGCTGTCTGTTGGTACCAGCGAAGTACAGA  1620
             |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1680  TGCGTGTGGCTATTCCGGCCGTCATCGTTGCGCTGTCTGTTGGTACCAGCGAAGTACAGA  1739

Query  1621  ACATGCTGAATGCGATTCCGGAAGTGGTGACCAATGGTCTGAATATCGCCGGTGGCATGA  1680
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1740  ACATGCTGAATGCGATTCCGGAAGTGGTGACCAATGGTCTGAATATCGCCGGTGGCATGA  1799

Query  1681  TCGTGGTGGTTGGTTATGCGATGGTTATCAACATGATGCGTGCTGGCTACCTGATGCCGT  1740
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1800  TCGTGGTGGTTGGTTATGCGATGGTTATCAACATGATGCGTGCTGGCTACCTGATGCCGT  1859

Query  1741  TCTTCTACCTCGGCTTCGTAACCGCAGCATTCACCAACTTTAACCTGGTTGCTCTGGGTG  1800
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1860  TCTTCTACCTCGGCTTCGTAACCGCAGCATTCACCAACTTTAACCTGGTTGCTCTGGGTG  1919

Query  1801  TGATTGGTACTGTTATGGCAGTGCTCTACATCCAACTTAGCCCGAAATACAACCGCGTAG  1860
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1920  TGATTGGTACTGTTATGGCAGTGCTCTACATCCAACTTAGCCCGAAATACAACCGCGTAG  1979


Query  1861  CCGGTGCGCCTGCTCAGGCAGCTGGTAACAACGATCTCGATAACGAACTGGACTAACAGG  1920
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1980  CCGGTGCGCCTGCTCAGGCAGCTGGTAACAACGATCTCGATAACGAACTGGACTAACAGG  2039

Query  1921  TGAGCGAAATGGTTGATACAACTCAAACTACCACCGAGAAAAAACTCACTCAAAGTGATA  1980
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2040  TGAGCGAAATGGTTGATACAACTCAAACTACCACCGAGAAAAAACTCACTCAAAGTGATA  2099
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Query  1981  TTCGTGACGTCTTCCTGCGTTCTAACCTCTTCCAGGGTTCATGGAACTTCGAACGTATGC  2040
             ||||||  ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2100  TTCGTGGCGTCTTCCTGCGTTCTAACCTCTTCCAGGGTTCATGGAACTTCGAACGTATGC  2159

Query  2041  AGGCACTGGGTTTCTGCTTCTCTATGGTACCGGCAATTCGTCGCCTCTACCCTGAGAACA  2100
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2160  AGGCACTGGGTTTCTGCTTCTCTATGGTACCGGCAATTCGTCGCCTCTACCCTGAGAACA  2219

Query  2101  ACGAAGCTCGTAAACAAGCTATTCGCCGTCACCTGGAGTTCTTTAACACCCAGCCGTTCG  2160
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2220  ACGAAGCTCGTAAACAAGCTATTCGCCGTCACCTGGAGTTCTTTAACACCCAGCCGTTCG  2279

Query  2161  TGGCTGCGCCGATTCTCGGCGTAACCCTGGCGCTGGAAGAACAGCGTGCTAATGGCGCAG  2220
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2280  TGGCTGCGCCGATTCTCGGCGTAACCCTGGCGCTGGAAGAACAGCGTGCTAATGGCGCAG  2339

Query  2221  AGATCGACGACGGTGCTATCAACGGTATCAAAGTCGGTTTGATGGGGCCACTGGCTGGTG  2280
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2340  AGATCGACGACGGTGCTATCAACGGTATCAAAGTCGGTTTGATGGGGCCACTGGCTGGTG  2399

Query  2281  TAGGCGACCCGATCTTCTGGGGAACCGTACGTCCGGTATTTGCAGCACTGGGTGCCGGTA  2340
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2400  TAGGCGACCCGATCTTCTGGGGAACCGTACGTCCGGTATTTGCAGCACTGGGTGCCGGTA  2459

Query  2341  TCGCGATGAGCGGCAGCCTGTTAGGTCCGCTGCTGTTCTTCATCCTGTTTAACCTGGTGC  2400
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2460  TCGCGATGAGCGGCAGCCTGTTAGGTCCGCTGCTGTTCTTCATCCTGTTTAACCTGGTGC  2519

Query  2401  GTCTGGCAACCCGTTACTACGGCGTAGCGTATGGTTACTCCAAAGGTATCGATATCGTTA  2460
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2520  GTCTGGCAACCCGTTACTACGGCGTAGCGTATGGTTACTCCAAAGGTATCGATATCGTTA  2579

Query  2461  AAGATATGGGTGGTGGCTTCCTGCAAAAACTGACGGAAGGGGCGTCTATCCTCGGCCTGT  2520
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2580  AAGATATGGGTGGTGGCTTCCTGCAAAAACTGACGGAAGGGGCGTCTATCCTCGGCCTGT  2639

Query  2521  TTGTCATGGGGGCATTGGTTAACAAGTGGACACATGTCAACATCCCGCTGGTTGTCTCTC  2580
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2640  TTGTCATGGGGGCATTGGTTAACAAGTGGACACATGTCAACATCCCGCTGGTTGTCTCTC  2699

Query  2581  GCATTACTGACCAGACGGGCAAAGAACACGTTACTACTGTCCAGACTATTCTGGACCAGT  2640
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2700  GCATTACTGACCAGACGGGCAAAGAACACGTTACTACTGTCCAGACTATTCTGGACCAGT  2759

Query  2641  TAATGCCAGGCCTGGTACCACTGCTGCTGACCTTTGCTTGTATGTGGCTACTGCGCAAAA  2700
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2760  TAATGCCAGGCCTGGTACCACTGCTGCTGACCTTTGCTTGTATGTGGCTACTGCGCAAAA  2819

Query  2701  AAGTTAACCCGCTGTGGATCATCGTTGGCTTCTTCGTCATCGGTATCGCTGGTTACGCTT  2760
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2820  AAGTTAACCCGCTGTGGATCATCGTTGGCTTCTTCGTCATCGGTATCGCTGGTTACGCTT  2879


Query  2761  GCGGCCTGCTGGGACTGTAAGACTGTTGTACACTACCGGGGCCTTTTGGCCCCGtttttt  2820
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2880  GCGGCCTGCTGGGACTGTAAGACTGTTGTACACTACCGGGGCCTTTTGGCCCCGTTTTTT  2939


Query  2821  tATCTGGAGGATTAATGACAATCACGGACCTGGTACTGATTCTTT  2865
             |||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2940  TATCTGGAGGATTAATGACAATCACGGACCTGGTACTGATTCTTT  2984
```

Figure5.5: *E. coli* ZSC113 and Wild Type gene sequence base comparison.

Query= *E. coli* ZSC113 sequence

Sbject = Subject (Wild type *E. coli*) sequence

Bases in red = Mutation site

Bases in black= Normal sequence

Escherichia coli ZSC113: Gene sequence

E.coli manX/manY/manZ

```
TTACCTTTCGAAATTTCTGCTAATCGAAAGTTAAATTACGGATCTTCATCACATAAAATA
ATTTTTTCGATATCTAAAATAAATCGCGAAACGCAGGGGTTTTTGGTTGTAGCCCTTATC  cloning primer
TGAATCGATTCGATTGTGGACGACGATTCAAAAATACATCTGGCACGTTGAGGTGTTAAC
GATAATAAAGGAGGTAGCAAGTGACCATTGCTATTGTTATAGGCACACATGGTTGGGCTG
CAGAGCAGTTGCTTAAAACGGCAGAAATGCTGTTAGGCGAGCAGGAAAACGTCGGCTGGA
TCGATTTCGTTCCAGGTGAAAATGCCGAAACGCTGATTGAAAAGTACAACGCTCAGTTGG
CAAAACTCGACACCACTAAAGGCGTGCTGTTTCTCGTTGATACATGGGGAGGCAGCCCGT
TCAATGCTGCCAGCCGCATTGTCGTCGACAAAGAGCATTATGAAGTCATTGCAGGCGTTA
ACATTCCAATGCTCGTGGAAACGTTAATGGCCCGTGATGATGACCCAAGCTTTGATGAAC
TGGTGGCACTGGCAGTAGAAACAGGCCGTGAAGGCGTGAAAGCACTGAAAGCCAAACCGG
TTGAAAAAGCCGCGCCAGCACCCGCTGCCGCAGCACCAAAAGCGGCTCCAACTCCGGCAA
AACCAATGGGGCCAAACGACTACATGGTTATTGGCCTTGCGCGTATCGACGACCGTCTGA
TTCACGGTCAGGTCGCCACCCGCTGGACCAAAGAAACCAATGTCTCCCGTATTATTGTTG
TTAGTGATGAAGTGGCTGCGGATACCGTTCGTAAGACACTGCTCACCCAGGTTGCACCTC
CGGGCGTAACAGCACACGTAGTTGATGTTGCCAAAATGATTCGCGTCTACAACAACCCGA  end of rev 2
AATATGCTGGCGAACGCGTAATGCTGTTATTTACCAACCCAACAGATGTAGAGCGTCTCG
TTGAAGGCGGCGTGAAAATCACCTCTGTTAACGTCGGTGGTATGGCATTCCGTCAGGGTA  primer for rev 3/fwd 2
AAACCCAGGTGAATAACGCGGTTTCGGTTGATGAAAAAGATATCGAGGCGTTCAAGAAAC
TGAATGCGCGCGGTATTGAGCTGGAAGTCCGTAAGGTTTCCACCGATCCGAAACTGAAAA
TGATGGATCTGATCAGCAAAATCGATAAGTAACGTATTGTGTTGATTATCACTCAGTTTT  end of fwd 1
CACACTTAAGTCTTACGTAAACAGGAGAAGTACAATGGAGATTACCACTCTTCAAATTGT
GCTGGTATTTATCGTAGCCTGTATCGCAGGTATGGGATCAATCCTCGATGAATTTCAGTT
TCACCGTCCGCTAATCGCGTGTACCCTGGTGGGTATCGTTCTTGGGGATATGAAAACCGG
TATTATTATCGGTGGTACGCTGGAAATGATCGCGCTGGGCTGGATGAACATCGGTGCTGC
AGTTGCGCCTGACGCCGCTCTGGCTTCTATCATTTCTACCATTCTGGTTATCGCAGGTCA
TCAGAGCATTGGTGCAGGTATCGCACTGGCAATCCCTCTGGCCGCTGCGGGCCAGGTACT
GACCATCATCGTTCGTACTATTACCGTTGCTTTCCAGCACGCTGCGGATAAGGCTGCTGA
TAACGGCAACCTGACAGCGATTTCCTGGATCCACGTTTCTTCTCTGTTCCTGCAAGCAAT
GCGTGTGGCTATTCCGGCCGTCATCGTTGCGCTGTCTGTTGGTACCAGCGAAGTACAGAA
CATGCTGAATGCGATTCCGGAAGTGGTGACCAATGGTCTGAATATCGCCGGTGGCATGAT
CGTGGTGGTTGGTTATGCGATGGTTATCAACATGATGCGTGCTGGCTACCTGATGCCGTT
CTTCTACCTCGGCTTCGTAACCGCAGCATTCACCAACTTTAACCTGGTTGCTCTGGGTGT  end of rev 1
GATTGGTACTGTTATGGCAGTGCTCTACATCCAACTTAGCCCGAAATACAACCGCGTAGC  primer for fwd 3
CGGTGCGCCTGCTCAGGCAGCTGGTAACAACGATCTCGATAACGAACTGGACTAACAGGT  primer for rev 2
GAGCGAAATGGTTGATACAACTCAAACTACCACCGAGAAAAAACTCACTCAAAGTGATAT  end of fwd 2
TCGTGGCGTCTTCCTGCGTTCTAACCTCTTCCAGGGTTCATGGAACTTCGAACGTATGCA
GGCACTGGGTTTCTGCTTCTCTATGGTACCGGCAATTCGTCGCCTCTACCCTGAGAACAA
CGAAGCTCGTAAACAAGCTATTCGCCGTCACCTGGAGTTCTTTAACACCCAGCCGTTCGT
GGCTGCGCCGATTCTCGGCGTAACCCTGGCGCTGGAAGAACAGCGTGCTAATGGCGCAGA
GATCGACGACGGTGCTATCAACGGTATCAAAGTCGGTTTGATGGGGCCACTGGCTGGTGT
AGGCGACCCGATCTTCTGGGGAACCGTACGTCCGGTATTTGCAGCACTGGGTGCCGGTAT
CGCGATGAGCGGCAGCCTGTTAGGTCCGCTGCTGTTCTTCATCCTGTTTAACCTGGTGCG
TCTGGCAACCCGTTACTACGGCGTAGCGTATGGTTACTCCAAAGGTATCGATATCGTTAA
AGATATGGGTGGTGGCTTCCTGCAAAAACTGACGGAAGGGGCGTCTATCCTCGGCCTGTT
TGTCATGGGGGCATTGGTTAACAAGTGGACACATGTCAACATCCCGCTGGTTGTCTCTCG
CATTACTGACCAGACGGGCAAAGAACACGTTACTACTGTCCAGACTATTCTGGACCAGTT
AATGCCAGGCCTGGTACCACTGCTGCTGACCTTTGCTTGTATGTGGCTACTGCGCAAAAA
AGTTAACCCGCTGTGGATCATCGTTGGCTTCTTCGTCATCGGTATCGCTGGTTACGCTTG
CGGCCTGCTGGGACTGTAAGACTGTTGTACACTACCGGGGCCTTTTGGCCCCGTTTTTTT
ATCTGGAGGATTAATGACAATCACGGACCTGGTACTGATTCTTTTCATCGCCGCACTCCT  cloning primer
GGCCTTCGCGATCTACGATCAGTTCATCATGCCCCGCCGTAACGGCCCCACCCTGCTGGC
AATTCCTTTGCTCCGGCGTGGTCGCATCGATAGCGTTAT
```

Figure 5.6: Gene sequence of *E. coli* ZSC113 Man PTS domains

Bases in red = Mutation site

Bases in green = Suggested not to encode a PTS domain by software

Bases in black= Normal sequence

Appendix III: StrataClone Vector

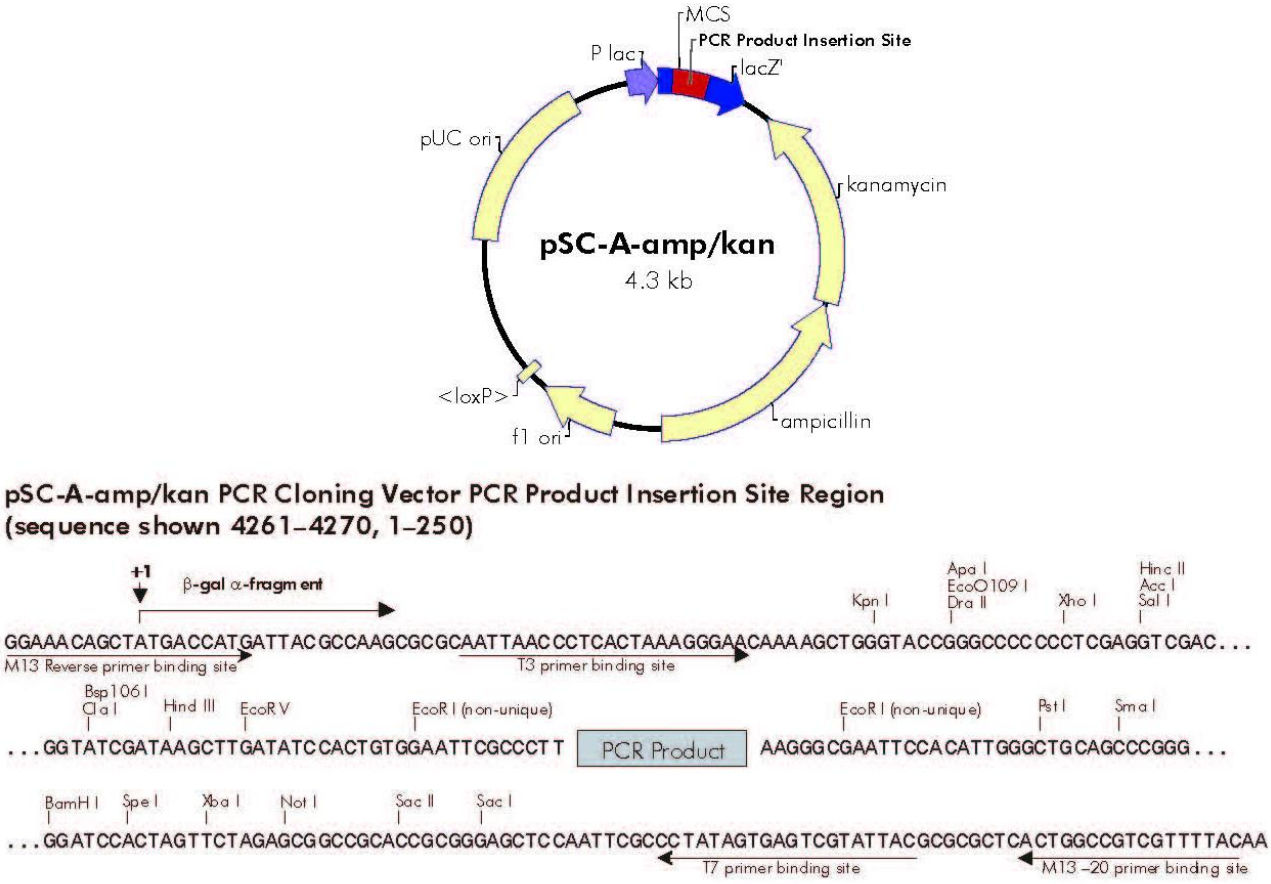

Figure 5.7: StrataClone PCR cloning vector pSC-A-amp/kan.

Appendix IV: Buffers and Solutions

Reaction Mix buffer for PCRs:

- 10x Buffer Mix: 500µl
- Adenine bases: 10µl
- Thymine bases: 10µl
- Guanine bases: 10µl
- Cytidine bases: 10µl
- Deionised water: 460µl

Total volume 1ml.

50x TAE (Tris-acetate) Buffer

- Tris: 242g dissolved in 500ml of deionised water
- Na_2EDTA: 250ml
- Glacial acetic acid: 57.1ml
- Deionised water: 192.9ml

Total volume 1L.

Appendix V: 1kb Hyperladder (Bioline)

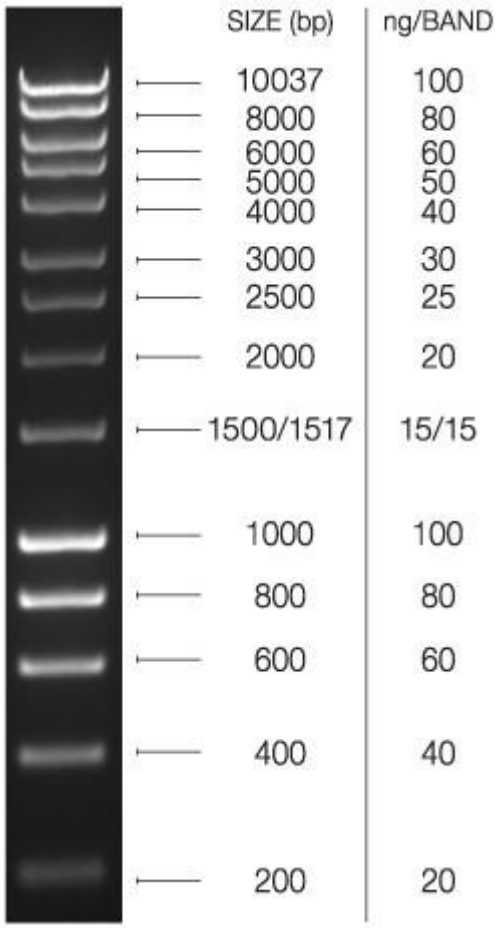

Figure 5.8: The 1 kb hyperladder used as a marker in all agarose electrophoresis gels.

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